β1 Integrin regulates convergent extension in mouse notogenesis, ensures notochord integrity and the morphogenesis of vertebrae and intervertebral discs

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MS TITLE: β1 integrin regulates convergent extension in mouse notogenesis ensuring notochord integrity and morphogenesis of vertebrae and intervertebral discs

AUTHORS: Shiny Shengzhen Guo, Tiffany YK Au, Sarah Wynn, Attila Aszodi, Danny Chan, Reinhard Faessler, and Kathryn Cheah

I have now received all the referees' reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

As you will see, the referees express considerable interest in your work, but have some significant criticisms and recommend a substantial revision of your manuscript before we can consider publication. There is general agreement between the referees about the novelty and potential importance of your work, andalso on the substantive issues that need to be addressed. In particular, I would draw your attention to the questions about the efficiency and timing of the cre mediated recombination and how this affects the interpretation of the phenotypes. In addition, it seems important to address the questions about the embryo culture experiments concerning the asymmetry in VANGL2 and whether integrin inhibition in the notochord is effective and disrupts PCP. All three referees were also concerned with the interpretation of the N-Cadherin staining in Figure 5.

If you are able to revise the manuscript along the lines suggested, which may involve further experiments, I will be happy receive a revised version of the manuscript. Your revised paper will be re-reviewed by one or more of the original referees, and acceptance of your manuscript will depend on your addressing satisfactorily the reviewers' major concerns. Please also note that Development will normally permit only one round of major revision.

We are aware that you may currently be unable to access the lab to undertake experimental revisions. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please attend to all of the reviewers' comments and ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion. I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

This paper describes the roles of beta1 integrin (Itgb1) in mouse notochord morphogenesis. To avoid peri-implantation lethality, the authors produced notochord-specific deletion mutants of the Itgb1 gene. Approximately half of the mutant mice showed abnormalities in vertebral body, intervertebral discs and nucleus pulposi at birth. In the mutant embryos, some parts of the notochord were fragmented and displaced. The mutant notochord showed multiple abnormalities including loss of fibronectin around the notochord, reduced convergent-extension movement of the node/notochord cells, and increased N-cadherin. Cell proliferation, cell death, and planar cell polarity were not affected. Authors established a notochord cell line, and showed that Itgb1 mutant cells lost attachment to fibronectin and have reduced cell motility.

This paper demonstrated requirement of Itgb1 in notochord development specifically for formation of the fibronectin sheath and morphogenetic cellular movement. These findings are novel and important for understanding the roles of integrin signaling and integrin mediated interaction with ECM during morphogenesis. The results are supported by detailed analyses of fixed embryos high-quality live imaging, and in vitro studies using the newly established notochord cell line. The major concern about this paper is that the efficiency of Cre-mediated gene knockout and its relationship with phenotype is not clearly described. Because of incomplete penetrance of the mutant phenotypes, such ambiguity makes interpretation of the results difficult.

Comments for the author

Major comments
1. Relationship among Itgb1 mutation, early notochord phenotypes, and late vertebral body/IVD/NP phenotypes is not clear.
a) Cre reporter expression by Foxa2mNE-Cre transgene is ‘mosaic’ (line 110), but expression of b1-integrin was ‘almost completely absent’ (line 119). This description suggests that mosaicism of the notochord cells is low (i.e. essentially all the cells are mutant). On the other hand, authors also described that “46% of the mutants are normal, possibly due to mosaic Cre activity (line 124).” This description gives an impression that mosaicism of the notochord cells is high (i.e. the efficiency of Itgb1 KO is low, and many wild-type cells are remaining). Which is correct? This is important, because, in the former case, the observed phenotype reflects the functions of b1-integrin, while in the latter case, the phenotype may reflect only some functions of the gene.
b) In Figure 1B, weak b1-integrin signal is present in the mutant notochord. Why was this data interpreted as ‘expression was almost completely absent’? Is it possible to quantitate the signals? If Cre activity is mosaic, then the expression of b1-integrin should become mosaic. Was such a mosaic pattern observed in immunostaining of the E9.5 notochord? If it was, what is the frequency of mutant cells?
c) Figure 3I. Is loss of FN sheath observed throughout the mutant notochord, or only in some regions which lack Itgb1? In the latter case, what fraction of the mutant notochord does show this phenotype?

2. Authors showed that the mutant notochord has stronger expression of N-cadherin (line 254). However, in Figure 5, it is difficult to clearly recognize N-cadherin signals from merged images. Single channel images are required. In addition, expression of Brachyury seems to be also stronger in the mutant notochord. Are these differences in signals significant or just a difference in staining/image acquisition conditions? Quantification of signals and statistical analyses are required.

Minor comments
1. Abbreviation for Brachyury. According to mouse genome informatics (http://www.informatics.jax.org/marker/MGI:98472), the gene symbol for Brachyury is T, and the synonyms are Bra, T1, and Tbxt. It might be better to use commonly used Bra instead of Bry as an abbreviation of brachyury.

Reviewer 2

Advance summary and potential significance to field

This paper demonstrates the role of B1 integrin singling within the notochord for morphogenesis of the notochord and subsequently for the spine in mouse. This paper nicely demonstrates that B1 integrin is required for convergent extension of the notochord. However, I do not agree with the data as is demonstrating a reduction of asymmetric VANGL2 localization in the notochord after B1 integrin blocking antibody treatment. Regardless, the loss of B1 integrin signaling is required to stimulate fibronectin accumulation within the notochord sheath, which likely causes a reduction in the ability of the notochord to hold together as a collective tissue and disrupts the typical axial positions notochord within the developing mouse embryo. This is very clearly demonstrated with multiple incidences of acellular regions or spatial displacement of notochord tissues at times away from floor plate along the D-V axis, or shifted away from the midline position. This loss of notochord stability is likely to have direct effects on the establishment of the NP, which is likely affecting the development and morphogenesis of segmented vertebrae and IVD tissues, leading to fusions and scoliosis. This paper strongly implicates the role of the notochord in patterning a well segmented vertebral column in a mouse model and has new implications on the relevance of notochord in human spine disorders, which has previously only been observed in zebrafish models. Whether defective spine morphogenesis is due to alterations of the differentiation status of the adjacent somite or sclerotome lineages was not revealed but would be important to more accurately test in this novel notochord-derived model of spine disorder in mouse.

Comments for the author

Figure 5: Analysis of the N-cadherin staining needs to be quantified, the merged figures do not represent the claim of enhanced N-cad staining. At the very less the merge images should be shown as individual greyscale images. The mean pixel intensity of the N-cad channel can then be normalized to the mean intensity of the same region of interested for the Bry channel.

Figure 6: To me the B1 integrin Ab. blockade continues to demonstrate asymmetric polarization of VANGL2 (white arrows; Fig. 6A), thus the conclusion that this approach disrupts PCP (line 272 and 273) is not well-supported by the data. Moreover, the quality of the immunostaining image in the B1 integrin blockade is not comparable to the IgM control and thus the quantification of percentage of cells with polarized VANGL2 seems a misleading way to quantify. Better to categorize the polarity of PCP components within each cell with respect to the midline using a ROSE diagram (for example: https://doi.org/10.1016/j.cub.2017.10.053). It may be helpful to utilize a double labeling approach for Fzd6 (R&D systems: AF1526) and Vangl2 (2G4 clone) to really observe the asymmetric polarity of PCP components within individual cells. This would greatly strengthen the model proposed and improve the interpterion of B1 integrin regulation of PCP signaling in the notochord.

Discussion:

407-408: The loss of notochord tissue is directly contributing to the loss of NP, which is solely derived from the notochord and not a reflection of altered differentiation of somite /sclerotome

lineages. Please clarify this point in the discussion. Signaling for the vertebrae could be the result of somatic differentiation deects, however these were not specifically addressed experimentally.

409-410: Suggestion that the notochord patterns sclerotome cell differentiation is a great one, perhaps this model could be addressed using in situ hybridization against Pax1/Pax9?

Reviewer 3

Advance summary and potential significance to field

This manuscript from the Cheah lab provides exquisite phenotyping of a new transgenic mouse model in which Integrin B1 is deleted in the notochord.
They report striking and convincing embryo phenotypes including development of fragmented notochords without fibronectin basement membranes surrounding them. Negative data they present – e.g. the persistent dorso-ventral neural tube pattering in areas not overlying a detectable notochord – is in itself very important. Their post-natal phenotyping demonstrates marked vertebral malformations (please see comments on controls and potential artifact). Some mechanistic investigations require additional explanation or controls for potentially confounding influences.

Comments for the author

1) The hypertrophic chondrocytes in Figure 1D seem larger in the mutant than the control (please provide scale bars). The authors need to include data demonstrating that their Sox9 promoter-based Cre driver is not active in even a subset of this Sox9-derived lineage. I am confident they will have considered this.

2) What are the lineage-traced cells which migrate laterally to the notochord in the live-imaging (especially towards the end) forming neural crest-like streams? How does Cre-recombination in these cells impact interpretation of post-natal phenotypes.

3) I am confused by timings presented. The authors state that the Cre they used "commences at E7.5-E8.0" and that the detectable "lifetime" of Integrin β1 is "~1.5 days in notochordal tissue. This means, Integrin β1 is lost ~E9 (~15 somites). In fact, they explain this may account for the lack of Vangl2 miss-localisation in embryos with ~6 somites (E8.5).
The live imaging started when embryos had ~1 somite so, assuming a somite is added every 2 hours, Integrin β1 is expected to still be present for at least 10 hours (6 somite stage) out of the 16 hours imaged. The majority of live imaging was therefore performed in embryos with persistent Integrin β1 able to direct localization of Vangl2 (if this is indeed a down-stream effect, see comment below). Persistent Integrin β1 expression in the mutant video would be consistent with the ability of lineage-traced cells to dart laterally to the notochord and migrate large distances during imaging.
If all this is correct, and I invite the authors to clarify if not, the differences they show in cell movement between control and mutant in the first ~400 minutes of Figure 4D cannot conceivably be due to loss of Integrin β1. The authors need to rethink, or re-explain, this analysis.

4) The live imaged sequences were registered using "non-moving reference dots". What and where were these dots? Based on the tracks provided it seems likely that they were rostral, which would interpret body axis elongation as caudal cell migration. It is essential to ensure the reference dots were a similar distance from the end of the tail in all embryos. To circumvent this confounding factor, the authors could register images using a point at the caudal-most end of the body. Both the control and mutant movies provided show beautiful convergence and extension.

5) Failure of convergent extension is argued based on morphological measurements of the notochord in flat-mounted embryos. Flat-mounting changes morphology, typically widening tissues. Mutant notochords without delimiting fibronectin are likely to spread out more. Please repeat this analysis in transverse cross-sections or non-flat-mounted whole mounts to account for this artefact, additionally providing information on whether the notochord cross-sectional area is different.

6) Histological artifact is unfortunately also likely in analyses of the gorgeous spinal histology shown. Kyphosis means each section will not run evenly through the center of each vertebra. You can see this clearly in the bottom right of figure 1D: the left-most IVD captures the end of the NP whereas the rightmost is lateral and does not show NPs. Were the authors able to account for this artefact?

7) The Vangl2 data presented is problematical. The in vivo data shows that conditional deletion of Itgb1 which causes the phenotypes they identified does not produce loss of Vangl2 planar polarization in the pit cell region. This is very elegantly demonstrated and excludes loss of Vangl2 polarisation in these cells as the underlying cause of their later phenotyes. However, the authors then use an inhibitory antibody to reduce the proportion of node cells with polarized Vangl2 and argue "The impact of the β1 integrin blocking… suggests that β1 integrin signaling is required for the establishment of PCP but not for its maintenance, which is consistent with a role in integrating PCP with convergent extension."
a. Given they demonstrate PCP polarization is established in their mutant embryos, how does Itgb1 deletion cause failure of CE (if CE does indeed fail, see points 4 and 5 above).
b. Having extensive experience of mouse embryo culture, I know that even molecules the size of phalloidin do not cross the yolk sac and amnion into mouse embryos. It is unlikely that IgM would. The authors need to demonstrate effective notochordal integrin inhibition to corroborate an effect on this tissue.
c. The tissue most directly impacted by the antibody is likely to be the yolk sac itself. The images provided show that the treated embryo has a smaller yolk sac and blood islands which seem less mature than in the control embryo. Lack of yolk sac expansion would eliminate resulting mechanical cues. The authors need to at least report somite stage and embryo length comparing the two groups, and should also report yolk sac projected area as a measure of its expansion in culture +/- antibody.

8) The reduction in cell and nuclear size in immortalized notochordal cells lacking Itgb1 in vitro is consistent with the literature yet nonetheless very dramatic. Do these immortalized cells express any other B integrins? Do nuclear/cell sizes also change in vivo?

9) Up-regulation of N-cadherin in Figure 5 is unconvincing. T, rather than N-cad, intensity seems much higher in the mutant than control. Please quantify this using internal intensity normalization (e.g. to DAPI) or provide additional evidence of N-cad up-regulation (e.g. RNA-level).

10) Whenever fluorescence images with merged channels are shown please always include the constituent channels independently, as done in Figure 6.
In some cases these can be provided as extended data.

11) Please specify the number of live-imaged embryos excluded because of perceived photo-toxicity and comment on the rate of development relative to roller bottle culture (which should be equivalent to in vivo).

12) Please include a statistical analysis methods section.

First revision

Author response to reviewers' comments

The following are our responses to the Reviewers' comments. Some data appended here in response are not incorporated into the revised manuscript, but we feel will help clarify the issues raised. These data are attached to the end of this response letter and labelled as Response Figures (1-6).

Reviewer 1

Advance Summary and Potential Significance to Field

This paper describes the roles of beta1 integrin (Itgb1) in mouse notochord morphogenesis. To avoid peri-implantation lethality, the authors produced notochord- specific deletion mutants of the Itgb1 gene. Approximately half of the mutant mice showed abnormalities in vertebral body, intervertebral discs and nucleus pulposi at birth. In the mutant embryos, some parts of the notochord were fragmented and displaced. The mutant notochord showed multiple abnormalities including loss of fibronectin around the notochord, reduced convergent-extension movement of the node/notochord cells, and increased N-cadherin. Cell proliferation, cell death, and planar cell polarity were not affected. Authors established a notochord cell line, and showed that Itgb1 mutant cells lost attachment to fibronectin and have reduced cell motility.

This paper demonstrated requirement of Itgb1 in notochord development, specifically for formation of the fibronectin sheath and morphogenetic cellular movement. These findings are novel and important for understanding the roles of integrin signaling and integrin mediated interaction with ECM during morphogenesis. The results are supported by detailed analyses of fixed embryos, high-quality live imaging, and in vitro studies using the newly established notochord cell line. The major concern about this paper is that the efficiency of Cre-mediated gene knockout and its relationship with phenotype is not clearly described. Because of incomplete penetrance of the mutant phenotypes, such ambiguity makes interpretation of the results difficult.

Reviewer 1 Comments for the Author...

Major comments
1. Relationship among Itgb1 mutation, early notochord phenotypes, and late vertebral body/IVD/NP phenotypes is not clear.
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b) In Figure 1B, weak b1-integrin signal is present in the mutant notochord. Why was this data interpreted as ‘expression was almost completely absent’? Is it possible to quantitate the signals? If Cre activity is mosaic, then the expression of b1-integrin should become mosaic. Was such a mosaic pattern observed in immunostaining of the E9.5 notochord? If it was, what is the frequency of mutant cells?

Response:
We thank the reviewer for appreciating the novelty of our findings. We apologise if the information provided and description of the Foxa2mNE-Cre transgene was not sufficient and clear. In the revised manuscript, we have provided additional information on the Cre line and its activity.

a) Cre reporter activity:

We now provide additional information on the Cre activity at E9.5 in ~400 embryos by two independent users. We show that apart from the mosaicism (Fig. S1B), depending on the breeder, there are 3 different types of Cre activities at E9.5 (Fig. S1C-S1D). As described on Page 7 (Line 116-125) in the revised manuscript, “Type-I (50%) shows strong activity in the entire notochord but also some activity in the tail bud from E9.5, which probably reflects the contribution of node cells to the tail bud (Cambray and Wilson., 2002, PMID: 12361976). The remaining 50% showed two types of notochord only activities: one which was strong (Type-II, 37%) and the other weak (Type-III, 13%) (Fig. S1D). Cre expression ceased around E14.5 and

GFP+ notochord descendants were found in the NP at adult stages (Au et al, manuscript in revision), which is consistent with published reports demonstrating a notochordal origin for cells in the NP (McCann et al., 2012, PMID: 22028328; Choi et al., 2008, PMID: 19035356)". The implication of these two types of Cre activity for the vertebral body and tail defects are discussed below and in the revised manuscript.

b) Mosaicism: With regard to the question on mosaicism, this was variable within each embryo and we have included information on this variation in the revised manuscript (Page 7-8 Line 130-138, and Fig.1B + Fig. S2A-B).

Due to the mosaic activity of the transgenic Cre, the impact on β1 integrin expression also displays mosaic pattern in the mutants. As shown in the Fig. 1B, β1 integrin signals at different notochordal levels in the same mutant showed different intensities, reflecting different degrees of mosaicism which were quantified (0 in Region-1, 17% in Region-2 and 38% in Region-3). Line profile analysis was performed within the T+ notochordal cells and in the surrounding neural tube to show the mosaic β1 integrin signals in the mutant notochord and the unaffected β1 integrin signals in the neural tube.

The mosaicism within an entire embryo was quantified at E9.5 (22~23 somites, n=3) (Fig.S2B). Mosaicism varied from ~16% to ~30% in different embryos.

The frequency of mosaicism was defined by the ratio between the cell numbers with mosaic β1 integrin expression and the total cell numbers that were T+DAPI+. Signal intensities were measured by ImageJ. Cells with an intensity <20 a.u. were grouped into "complete knockout", >20 a.u. were grouped into "mosaic expression" (illustrated in Fig.S2A and also see in Materials and Methods).

c) Phenotype interpretation:
For the data shown in this paper we did not screen for a particular activity type (Type I or Type II) of Cre for the studies on early notochord development (E8.5- 10.5). Since both Cre types express strongly in the notochord and somites developed normally before E10.5 (Fig.S7A-C and see below), the interpretation of the early phenotype should be robust.

However reviewing the data on Cre activity has prompted us to think more carefully about the interpretation with regard to the tail vertebral phenotype that was observed in the postnatal conditional mutants. As it is not possible to ascribe the tail vertebrae phenotype at birth solely to Cre activity in the notochord, we have revised our discussion on the origin of tail vertebral defects. However the influence of the notochord on development of the vertebrae cannot be excluded. Lineage tracing for the cells in the node of the late-streak stage embryo (~E7.5), have been shown to contribute to the anterior midline mesoderm (also known as the head process and/ or anterior notochord underlying the mid- to hindbrain). Node cells from more advanced embryos (E7.5-7.75) (likely to be posterior pit + crown cells) also contribute to the notochord in the upper trunk (Tam et al., 1997, PMID: 9598345; Kinder, et al., 2001, PMID: 11566865; Robb and Tam, 2004, PMID: 15271300; Yamanaka et al., 2007, PMID: 18061569). In addition whether descendants of tail bud cells can contribute to thoracic vertebrae is an open question, especially since fate mapping studies using 9.5 d.p.c.-13.5 d.p.c tail bud cells show contribution to more posterior somites (Tam and Tan, 1992, PMID: 1425350). Furthermore, grafting studies have shown that tail bud cells do not incorporate well to the axis, and at best tend to contribute to short stretches of somites (Cambray and Wilson, 2002, PMID: 12361976). Therefore since abnormal vertebrae were also observed in thoracic regions postnatally (Fig.2B), and displaced notochord segments at the level of future trunk vertebrae were found at E12.5 (Fig.S5), a possible influence of the notochord cannot be excluded. We have therefore revised the Discussion section on this aspect (Page 22, Line 468-481).

c) Figure 3I. Is loss of FN sheath observed throughout the mutant notochord, or only in some regions which lack Itgb1? In the latter case, what fraction of the mutant notochord does show this phenotype?

Response:
The pattern of FN loss/reduction was also mosaic. Three examples are shown in Response

Figure 1 (Pair-1 to Pair-3). The reduction/loss of FN sheath assembly largely correlated with loss of β1 integrin expression. For example in Pair-3, in regions with high mosaicism, FN is still recruited in the adjacent notochord structure. In regions with low mosaicism like in Pair-1 and Pair-2, FN sheath formation is consistently reduced or lost. Please note in pair-2, the mutant notochord (yellow arrows), which still show weak but sharp β1 integrin signal, also displayed a sharp FN sheath formation, suggesting β1 integrin loss is the reason for the weakened/reduced FN sheath formation.

FN assembly was analyzed at E9.5 via cross sections of 5 control and 7 mutant embryos. For each embryo, 4 sections with 8 different levels were analyzed (anterior + posterior). 5/7 mutants showed obvious defects with either D-V, L-R deviation or interrupted notochord. 4 out of these 5 embryos displayed loss or reduced FN sheath assembly in some regions. Thus the observed ratio of FN loss/reduction is 4/7= 57%. Please refer to Response Table 1 for more details.

These information was updated in the revision on Page 36, Line 908-911.

2. Authors showed that the mutant notochord has stronger expression of N-cadherin (line 254). However, in Figure 5, it is difficult to clearly recognize N-cadherin signals from merged images. Single channel images are required. In addition, expression of Brachyury seems to be also stronger in the mutant notochord. Are these differences in signals significant or just a difference in staining/image acquisition conditions? Quantification of signals and statistical analyses are required.

Response:
Thank you for raising this question. To clarify, the entire view for the whole notochordal plate, images for the separate grey channels for N-cadherin, T and DAPI are provided in the revised manuscript (Figure 6). Since we do not know whether loss of β1 integrin would impact on T expression, we normalized the N-cadherin signal intensities to the DAPI (which are also T+) signals. Statistical analysis of the data from 3 staged-matched embryo pairs in the notochordal region (Fig. 6C) showed a significant increase in N-cadherin (P<0.0001, two-tailed student t-test). An improved Figure (Figure 6) on samples with comparable T expression is included in the revised manuscript. The new figure from the original submission with stronger Brachyury expression is also enclosed for reference (Response Figure 2).
Furthermore we have included cross section staining of N-cadherin at E9.5 (Fig. 6B and quantified in 6C), which further demonstrates the enhanced N-cadherin expression in the mutant embryos.
Please note that the imaging acquisition settings for the targeting signals (N- cadherin) and the DAPI signals used for normalization were exactly the same for the paired embryos analyzed.
The original LSM files have been uploaded for reference (Con1 & Mu1 for the repeat shown in the revision as Figure 6; Con3 & Mu3 for the repeat shown in the initial submission as Response Figure 2). !!Due to the size limitation, we cannot upload all LSM files (8.2M each X12 files=~100M). Only one region from each embryo was submitted (we tried several times, realized that 4 LSM files are the maximal acceptable size load together with the other files).

The text has been updated accordingly in the revision (Page 14, Line 292-296).

Minor comments
1. Abbreviation for Brachyury. According to mouse genome informatics (http://www.informatics.jax.org/marker/MGI:98472), the gene symbol for Brachyury is T, and the synonyms are Bra, T1, and Tbxt. It might be better to use commonly used Bra instead of Bry as an abbreviation of brachyury.

Response:
Thank you for the comment. Since by convention protein is in upper case not italics, and the official gene symbol for mouse brachyury is *T*, we have changed all reference from brachyury to T (for protein) and *T* for the gene.

Reviewer 2 Advance Summary and Potential Significance to Field...

This paper demonstrates the role of B1 integrin singling within the notochord for morphogenesis of the notochord and subsequently for the spine in mouse. This paper nicely demonstrates that B1 integrin is required for convergent extension of the notochord. However, I do not agree with the data as is demonstrating a reduction of asymmetric VANGL2 localization in the notochord after B1 integrin blocking antibody treatment. Regardless, the loss of B1 integrin signaling is required to stimulate fibronectin accumulation within the notochord sheath, which likely causes a reduction in the ability of the notochord to hold together as a collective tissue and disrupts the typical axial positions notochord within the developing mouse embryo. This is very clearly demonstrated with multiple incidences of acellular regions or spatial displacement of notochord tissues at times away from floor plate along the D-V axis, or shifted away from the midline position. This loss of notochord stability is likely to have direct effects on the establishment of the NP, which is likely affecting the development and morphogenesis of segmented vertebrae and IVD tissues, leading to fusions and scoliosis. This paper strongly implicates the role of the notochord in patterning a well segmented vertebral column in a mouse model and has new implications on the relevance of notochord in human spine disorders, which has previously only been observed in zebrafish models. Whether defective spine morphogenesis is due to alterations of the differentiation status of the adjacent somite or sclerotome lineages was not revealed but would be important to more accurately test in this novel notochord-derived model of spine disorder in mouse.

Reviewer 2 Comments for the Author...

1) Figure 5: Analysis of the N-cadherin staining needs to be quantified, the merged figures do not represent the claim of enhanced N-cad staining. At the very less the merge images should be shown as individual greyscale images. The mean pixel intensity of the N-cad channel can then be normalized to the mean intensity of the same region of interested for the Bry channel.

Response:
Thank you for the suggestion. Please refer to our response to Reviewer-1 (Question-2) and the new assembled Figure 6 in the revision, and the Response Figure 2 for more details.

2) Figure 6: To me the B1 integrin Ab. blockade continues to demonstrate asymmetric polarization of VANGL2 (white arrows; Fig. 6A), thus the conclusion that this approach disrupts PCP (line 272 and 273) is not well-supported by the data. Moreover, the quality of the immunostaining image in the B1 integrin blockade is not comparable to the IgM control and thus the quantification of percentage of cells with polarized VANGL2 seems a misleading way to quantify. Better to categorize the polarity of PCP components within each cell with respect to the midline using a ROSE diagram (for example: https://doi.org/10.1016/j.cub.2017.10.053). It may be helpful to utilize a double labeling approach for Fzd6 (R&D systems: AF1526) and Vangl2 (2G4 clone) to really observe the asymmetric polarity of PCP components within individual cells. This would greatly strengthen the model proposed and improve the interpretation of B1 integrin regulation of PCP signaling in the notochord.

Response:

a) Thank you for the question. It is unfortunate that the current Cre tool does not express early enough and in addition is not expressed in the pit cells of the node (which gives rise to the anterior notochord (Sulik K, et al., 1994. PMID: 7881129) and so we are unable to test for a PCP phenotype in the conditional mutants. However the ex vivo impact of the ß1 integrin blocking antibody on the frequency of node cells with proper asymmetric localization of VANGL2 in developing embryos suggests a link between the integrin signaling and the establishment of the PCP pathway.

In regard to the questions about the blocking antibody experiments, we hope the following information will address the reviewer's concern. After overnight embryo culture, the staining from the ß1 integrin blocking group always showed less defined signals for the markers analyzed, including both VANGL2, Phalloidin (F-actin) and N- cadherin. That is possibly the reason for the impression that the staining quality is not comparable between control and blocking group. Although all data for the three repeats are not included in the revised manuscript, we provide

the images of these repeats in Response Figure 3 (Pair-1 to Pair-3) for reference.

b) Thank you for the suggestion to use ROSE diagrams to represent the orientation of VANGL2 signals. The measuring details and definition of the asymmetric localization are illustrated and data plotted using ROSE diagram are shown in revision as Fig. S12C. Possibly due to the blocking efficiency is not 100%, few cells from the group treated with the blocking antibodies still displayed asymmetric VANGL2 localization, in which the orientation was comparable to the control group (Fig. S12C-S12E), but a clear significant reduction was observed in the percentage of the cells expressing asymmetric VANGL2 in the blocking antibody samples (Fig. 7A-7B, $p < 0.003$).

The text has been updated in the revision (Page 15-16, Line 311-321).

c) As the reviewer must be aware, there are few good antibody reagents that can reveal changes in PCP in cells within tissues. We found that the VANGL2 antibody provided by Dr. Kelley was the only one that could detect robustly, polarized localisation as a readout of PCP. This antibody has been cited in other publications on PCP (Montcouquiol et al., 2006, PMID: 16687519; Mahaffey et al., 2013, PMID: 23406901). To broaden our tests, we tested antibodies against many other PCP markers for asymmetric localization using embryos and also in tissues in which PCP is known to have important roles such as for chondrogenesis. Unfortunately none of those reagents worked. As shown in Response Figure 4, only VANGL2 antibody showed a clear and sharp anterior localization in the pit cells of the node.

Therefore, as working tools for the analysis suggested are not available, we are unable to perform the additional assays. We hope that the reviewer will accept this limitation.

3) Discussion:

407-408: The loss of notochord tissue is directly contributing to the loss of NP, which is solely derived from the notochord and not a reflection of altered differentiation of somite /sclerotome lineages. Please clarify this point in the discussion. Signaling for the vertebrae could be the result of somatic differentiation defects, however these were not specifically addressed experimentally.

Response:
Thank you for this important question. We have clarified that loss of NP is the direct consequence of the notochord loss at early embryonic stages in the discussion (Page 17-18, Line 367-373). With regard to the question on the impact of the notochord on patterning the axial skeleton please see our response to Reviewer-1 (Question-1) above and **also below**.

d) 409-410: Suggestion that the notochord patterns sclerotome cell differentiation is a great one, perhaps this model could be addressed using in situ hybridization against Pax1/Pax9?

Response:
With regard to possible defects in somitic differentiation, we tested for abnormal somite development by examining the expression of *Uncx4.1* and *Pax1* by *WISH*. As shown in Fig. S7A-C, somite differentiation was normal until E10.5, at which stage *Pax1* expression was interrupted in the caudal end (tail) in mutants. Consistent with these effects, obvious tail abnormalities were observed from E12.5 (Fig. S8). As described in our response to Reviewer-1 (Question-1), given our further examination of the pattern of cre activity, we cannot with certainty ascribe the vertebral defects only to the notochord but also consider that defects in differentiation of the sclerotome and prevertebrae may contribute postnatal tail phenotype. However the contribution of the notochord in addition cannot be excluded since vertebral body defects were seen in the thoracic vertebrae at P10 (Fig. 2B) and abnormal looking developing prevertebrae were seen adjacent to the displaced notochord at E12.5 (Fig. S5). Further clarification of the separable and combined impact will require detailed inducible CreERT mediated lineage tracing in the Type-I and Type-II transgenics, which would require the generation of new transgenic reagents and is beyond the scope of this paper.

We have therefore updated these observation in the results (Page 10, Line 191-197) and amended the Discussion of these results to reflect this (Page 22, Line 468-481).

Reviewer 3 Advance Summary and Potential Significance to Field...

This manuscript from the Cheah lab provides exquisite phenotyping of a new transgenic mouse model in which Integrin B1 is deleted in the notochord. They report striking and convincing embryo phenotypes including development of fragmented notochords without fibronectin basement membranes surrounding them. Negative data they present – e.g. the persistent dorso-ventral neural tube patterning in areas not overlying a detectable notochord – is in itself very important. Their post-natal phenotyping demonstrates marked vertebral malformations (please see comments on controls and potential artifact). Some mechanistic investigations require additional explanation or controls for potentially confounding influences.

Reviewer 3 Comments for the Author...

1) The hypertrophic chondrocytes in Figure 1D seem larger in the mutant than the control (please provide scale bars). The authors need to include data demonstrating that their Sox9 promoter-based Cre driver is not active in even a subset of this Sox9-derived lineage. I am confident they will have considered this.

Response:
Thank you for the query.
a) We have examined the staining of hypertrophic chondrocytes from the same litter, and as illustrated in Response Figure 5, we can conclude that the slightly different sizes of the hypertrophic chondrocytes are within the normal variations between different animals. Different planes of section angles is a possible reason for the "larger" impression. Scale bars have been included in the new Fig.2B.
b) Cross (E11.5) or sagittal (E12.5) sections were stained with either ß1 integrin alone or in combination with SOX9 (Fig.S3A-B). Although weak ß1 integrin expression still can be observed due to the Cre mosaicism or the long half-life of this protein, it clearly shows that the Cre is working only in the notochord or notochord remnants, but not in the surrounding SOX9+ cells in the developing cartilage.

The text has been updated on Page 8, Line 138-143.

2) What are the lineage-traced cells which migrate laterally to the notochord in the live-imaging (especially towards the end) forming neural crest-like streams? How does Cre-recombination in these cells impact interpretation of post-natal phenotypes.

Response:
We are not certain about the identity of the few GFP cells migrating laterally to the notochord in the videos that the reviewer refers to. We can only speculate as to their identity. Cre activity as reflected by GFP expression can be seen at 0 somite stage in the node and notochord (Fig.S1B). This is the stage when notochord precursor cells separate and leave the endoderm on the ventral surface of the embryo to form the rod-like notochord, a stage which is not well characterized (Balmer et al., 2016, PMID: 26845388). It has also been reported from cell labelling experiments that cells of the dorsal epithelia adjacent to the node can be found later in the notochord (Beddington, 1981, PMID: 7310311). It has been reported that emerging visceral endoderm cells clustered at the midline, align on either side of the notochordal plate and the node (Balmer et al., 2016, PMID: 26845388). These events occur at early somite stages. As live imaging were performed on embryos at similar time windows from ~1s to ~8s, we speculate that the GFP cells referred to, could be these cells. Since these cells are known to express *Foxa2*, this is a possibility.

With regard to the implication of Cre activity in these cells for the postnatal phenotype, we have discussed the contribution of later tail bud Cre activity in our response to Reviewer-1 (Question-1) above.

3) I am confused by timings presented. The authors state that the Cre they used "commences at E7.5-E8.0" and that the detectable "lifetime" of Integrin ß1 is "~1.5 days in notochordal tissue. This means, Integrin ß1 is lost ~E9 (~15 somites). In fact, they explain this may account for the lack of Vangl2 miss-localisation in embryos with ~6 somites (E8.5). The live imaging started when embryos had ~1 somite so, assuming a somite is added every 2 hours, Integrin ß1 is expected to still be present for at least 10 hours (6 somite stage) out of the 16 hours imaged. The majority of live imaging was therefore performed in embryos with persistent Integrin ß1 able to direct localization of Vangl2 (if this is indeed a down-stream effect, see comment below). Persistent Integrin ß1 expression in the mutant video would be consistent with the ability of lineage-traced cells to dart laterally to the notochord and migrate large distances during imaging. If all this is correct, and I invite the authors to clarify if not, the differences they show in cell movement between control and mutant in the first ~400 minutes of Figure 4D cannot conceivably be due to loss of Integrin ß1. The authors need to rethink, or re-explain, this analysis.

Response:
Thank you for the insightful analyses of our data. We apologise for incomplete clarity.

With regard to persistence of ß1 integrin protein, it is well established that ß1 integrin protein has a relatively long "half-life" until it is completely degraded. The half-life of ß1 integrin protein differs among different tissues/cells and can be more than 20-24 h (Brakebusch et el., 2000, PMID: 10921880; Böttcher et al., 2012, PMID: 22561348). Moreover in the skin, reduced levels of ß1 integrin have a phenotypic impact, suggesting a dosage requirement for the protein for full function (Brakebusch et el., 2000, PMID: 10921880). In the notochord, we observed a half- life of around 1.5 days.

In regard to the timing of loss of ß1 integrin and persistent expression, from Fig. 1A, ß1 integrin expression was significantly decreased comparing to control notochordal cells at E8.0 with ~1 somite, suggesting ß1 integrin gene was already inactivated.
The residual staining we detected can result from Cre mosaicism, or reflect stable proteins translated before Cre-mediated deletion at E8.0 or persisting long-lived integrin that are recycled back to the cell surface. Although residual ß1 integrin was detected, the notochord was already interrupted at E8.25 (2~3 somites, Fig. 3A and Fig.S7A, red arrows), suggesting this degree of reduction of ß1 integrin protein level is already sufficient to cause a defect.

Therefore with regard to the relationship between persistent ß1 integrin expression in the mutant and migration, please note that there was reduced ß1 integrin expression in the tracking region (crown cells) (Fig.S10, white arrowheads).

The text has been updated in Page 14, Line 289-291.

4) The live imaged sequences were registered using "non-moving reference dots". What and where were these dots? Based on the tracks provided it seems likely that they were rostral, which would interpret body axis elongation as caudal cell migration. It is essential to ensure the reference dots were a similar distance from the end of the tail in all embryos. To circumvent this confounding factor, the authors could register images using a point at the caudal-most end of the body. Both the control and mutant movies provided show beautiful convergence and extension.

Response:
We apologise for the confusion. The video alignment was done manually by aligning the images one by one, which was acquired every 10 minutes. Between every two time points, there were always some slow-moving or non-moving cells serving as the reference dots, together with the help of the embryo midline axis and the morphologies of cells tracked, the entire video was constructed. Although at the end, mutants also managed to converge and extend the notochord towards midline axis, it is clear the efficiency is reduced comparing to control embryos. Thus we observed a wider notochord width in the mutants.

These information has been updated in the Materials and Methods (Page 26, Line 571- 572).

5) Failure of convergent extension is argued based on morphological measurements of the notochord in flat-mounted embryos. Flat-mounting changes morphology, typically widening tissues. Mutant notochords without delimiting fibronectin are likely to spread out more. Please repeat this analysis in transverse cross-sections or non-flat-mounted whole mounts to account for this artefact, additionally providing information on whether the notochord cross-sectional area is different.

Response:
It is unlikely that the observed tissue widening is caused by an artifact of flat- mounting. It is clear the flat-mounted embryos were nicely mounted without folding. It is highly unlikely that a region with only 2 cells/row will become 4~5 cells/row by flat-mounting.

In support of this, we inspected the staining on the cross sections of the notochord stained with Brachyury (T) antibody at E9.5. Counting of notochord cells per sections for these embryos, clearly show that the notochord cell number per cross section was significantly increased in the mutants (Response Figure 6 and quantified in Fig. 5D) and is consistent with a wider notochord. We have enclosed several example images to illustrate this point (Response Figure 6).

Text has been updated accordingly on Page 13, Line 268-269.

6) Histological artifact is unfortunately also likely in analyses of the gorgeous spinal histology shown. Kyphosis means each section will not run evenly through the center of each vertebra. You can see this clearly in the bottom right of figure 1D: the left- most IVD captures the end of the NP whereas the rightmost is lateral and does not show NPs. Were the authors able to account for this artefact?

Response:
Thank you for the enquiry. We were aware of this possible artefact when analyzing this phenotype. Thus the entire spine was divided into several parts, including thoracic, lumbar, sacral and tail levels, and embedded separately. The entire block was sectioned, and every 5th section was stained. An example is shown in Fig. S4 where the NP is clearly missing from the IVD. And a summary of the incidence of absent NP is included in the revised manuscript as Supplementary Table 1.

Text has been updated on Page 8-9, Line 159-162.

7) The Vangl2 data presented is problematical. The in vivo data shows that conditional deletion of Itgb1 which causes the phenotypes they identified does not produce loss of Vangl2 planar polarization in the pit cell region. This is very elegantly demonstrated and excludes loss of Vangl2 polarisation in these cells as the underlying cause of their later phenotyes. However, the authors then use an inhibitory antibody to reduce the proportion of node cells with polarized Vangl2 and argue “The impact of the ß1 integrin blocking… suggests that ß1 integrin signaling is required for the establishment of PCP but not for its maintenance, which is consistent with a role in integrating PCP with convergent extension.”
a. Given they demonstrate PCP polarization is established in their mutant embryos, how does Itgb1 deletion cause failure of CE (if CE does indeed fail, see points 4 and 5 above).

Response:
With respect to the relationship between ß1 integrin and VANGL2 in our mutants at E8.5, please note that PCP is established in the pit cells which give rise to the anterior notochord (Hashimoto et al. 2010, PMID 20098415; Song et al., 2010, PMID 20562861). Since our Cre line has no activity in these cells, it is logical that we did not see defective VANGL2 polarization in pit cells within the node of the *Itgb1*$^{\Delta ND}$ mutants. Moreover, *Vangl2* is not expressed in the developing notochord (Torban et al., 2008, PMID 18296642), therefore we would not expect to see a change in VANGL2 polarization in the notochord of mutants and the differences in cell migration cannot be attributed to aberrant VANGL2.

Since *Vangl1* (which is expressed in the notochord) mutants do not display an embryonic PCP phenotype (Torban et al., 2008, PMID 18296642; Song et al., 2010, PMID 20562861), the convergent extension and altered migration may not be caused by a Vangl-mediated PCP defect,

consistent with other instances where the relationship between PCP and convergent extension is not absolute (Tada and Heisenberg, 2012, PMID 23048180). However the results of the blocking antibody experiments do raise the possibility that β1 integrin is important for the early establishment of PCP in the node which could impact on the developing notochord, although we cannot prove this in vivo. We have reflected on these possibilities in the revised Discussion (Page 20-21, Line 424-446). However if the reviewer prefers we are willing to remove the blocking antibody data from the manuscript.

b. Having extensive experience of mouse embryo culture, I know that even molecules the size of phalloidin do not cross the yolk sac and amnion into mouse embryos. It is unlikely that IgM would. The authors need to demonstrate effective notochordal integrin inhibition to corroborate an effect on this tissue.

Response:
Thank you for the comment. Although large molecules may not diffuse easily into embryos, this is not the case for antibodies with Fc regions. During gestation, maternal IgG is transmitted from mother to young in mammals to protect the young until its immune system is fully developed. The difference between IgG and IgM is that IgG carries only one Fc domain, but IgM carries 5 Fc domains (see the following):

Roverts et al. showed that Fc receptor is expressed in the surface of embryo yolk sac (Roberts et al., 1990, PMID 2146275);
Kim et al. showed that Fc receptor in the yolk sac is required for IgG transport to fetus (Kim et al., 2009, PMID 19234152);
Merad and Wild showed that IgM can be transported into the fetus (Merad and Wild, 1992, PMID 1635915).

c. The tissue most directly impacted by the antibody is likely to be the yolk sac itself. The images provided show that the treated embryo has a smaller yolk sac and blood islands which seem less mature than in the control embryo. Lack of yolk sac expansion would eliminate resulting mechanical cues. The authors need to at least report somite stage and embryo length comparing the two groups, and should also report yolk sac projected area as a measure of its expansion in culture +/- antibody.

Response:
Thank you for the suggestions. In the attached Response Figure 3 (Pair 1 to Pair 3) and in Fig. S12A-S12B, we have indicated somite numbers at the end of culture in the images. The measurements show that there is no difference of the embryo length and the yolk sac area (p= 0.9779 and 0.4487, respectively with paired student t-test). Since no blood islands were visible, no measurements were performed for this parameter.

The text has been updated in the revision (Page 15, Line 311-312).

8) The reduction in cell and nuclear size in immortalized notochordal cells lacking Itgb1 in vitro is consistent with the literature yet nonetheless very dramatic. Do these immortalized cells express any other B integrins? Do nuclear/cell sizes also change in vivo?

Response:
Thank you for the observation and question. A review of the *in vivo* tissue staining patterns at different embryonic stages showed no change of the nuclear/cell sizes.

In regard to integrins expressed, the expression profile of integrins in immortalized notochord cells was analyzed by FACS (Fig. S13C). The data show that the most abundant integrins are the FN receptor, a5β1, followed by VN receptor, avβ3. LN receptor a6β1 is also expressed in these cells but collagen receptors, such as a1 integrin is very low. The integrin expression profile correlates well with the adhesion assay showing these cells adhere well on FN and VN (but less potently on collagen I and laminin Fig.8A). Since avβ3 is still expressed when β1 is deleted, the mutant cells can still adhere to VN, although much less comparing to control.

We have revised the text accordingly in the revision (Page 16, Line 330-333 and Page 27- 28,

Line 596-610).

9) Up-regulation of N-cadherin in Figure 5 is unconvincing. T, rather than N-cad, intensity seems much higher in the mutant than control. Please quantify this using internal intensity normalization (e.g. to DAPI) or provide additional evidence of N-cad up-regulation (e.g. RNA-level).

Response:
Thank you for the suggestion. Separate grey channels for each marker are provided in the new assembled Fig. 6. N-cadherin signal intensities have been quantified and normalized to the T-positive DAPI signals. Please refer to our response to Reviewer-1 (Question-2) for more details.

We also analyzed N-cadherin expression in the mutants from E9.5 embryos (Fig. 6B and quantified in 6C, n=3 for each genotype). The impact on N-cadherin expression was also examined in the ß1 knockout notochord cells. As shown in Fig. 8F, N-cadherin expression is dramatically increased in the mutant cells.

Text has been updated on Page 14, Line 292-296 and Page 17, Line 347-348.

10) Whenever fluorescence images with merged channels are shown please always include the constituent channels independently, as done in Figure 6.
In some cases these can be provided as extended data.

Response:
We have updated the images in the revision. Fig.1A-B with single channel in grey for ß1 integrin expression, Fig.4C for active Caspase signals, Fig.4J for ß1 integrin, Fig. 4K for both LN and ß1 integrin in grey, Fig.6 for N-cadherin, T and DAPI signals in grey.

11) Please specify the number of live-imaged embryos excluded because of perceived photo-toxicity and comment on the rate of development relative to roller bottle culture (which should be equivalent to in vivo).

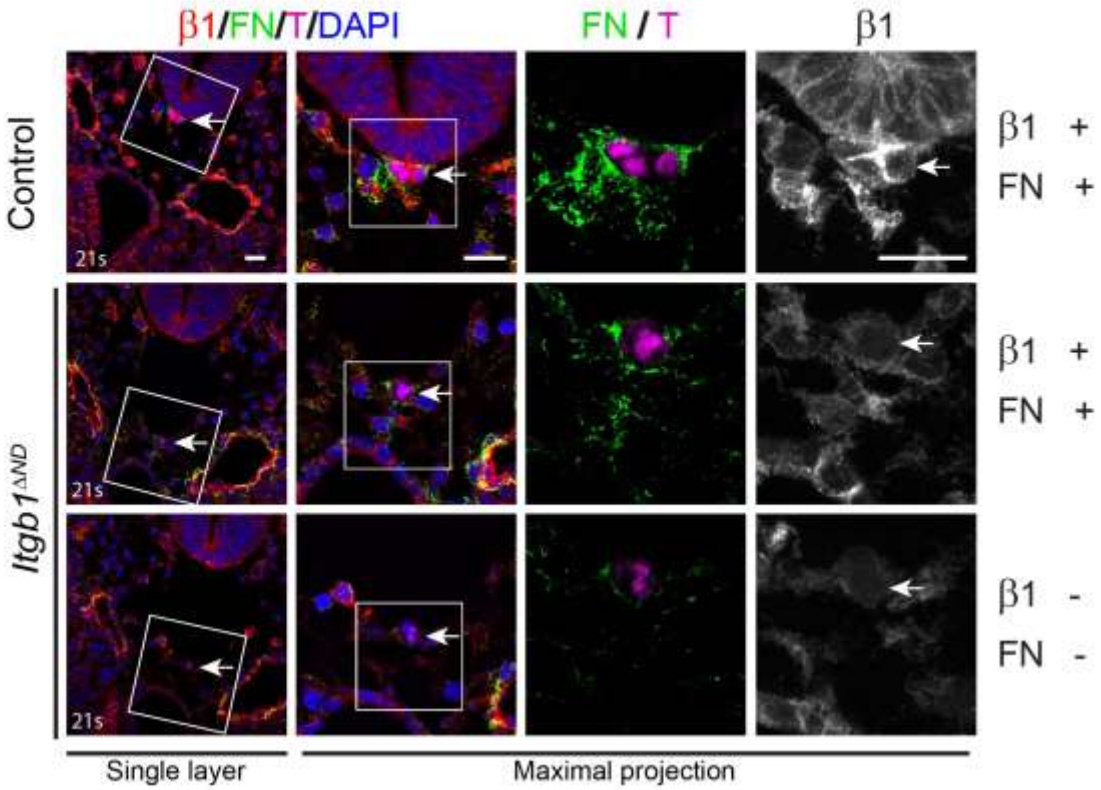
Response:
For overnight live-imaging, the experiments were performed as follows: total 7 times for control embryos (including tests) and 3 times for mutant embryos. In total only one control embryo was discarded at the end point due to an obvious shrinkage of the yolk sac.
For control embryos, after 16.8 hours of culture, microscopic examination showed somites usually reached 8 ~ 9s, depending on the starting age which was normally 1~2s. The growth rate is therefore 16.8hrs/7s=2.4hrs/s, which is slightly slower compared to the *in vivo* situation.

12) Please include a statistical analysis methods section.

Response:
Thank you for the suggestion. We have included a section on statistics in the revision (Page 30, Line 648-653).

Response Figure 1-Pair-1

Pair-1 with defective D-V alignment

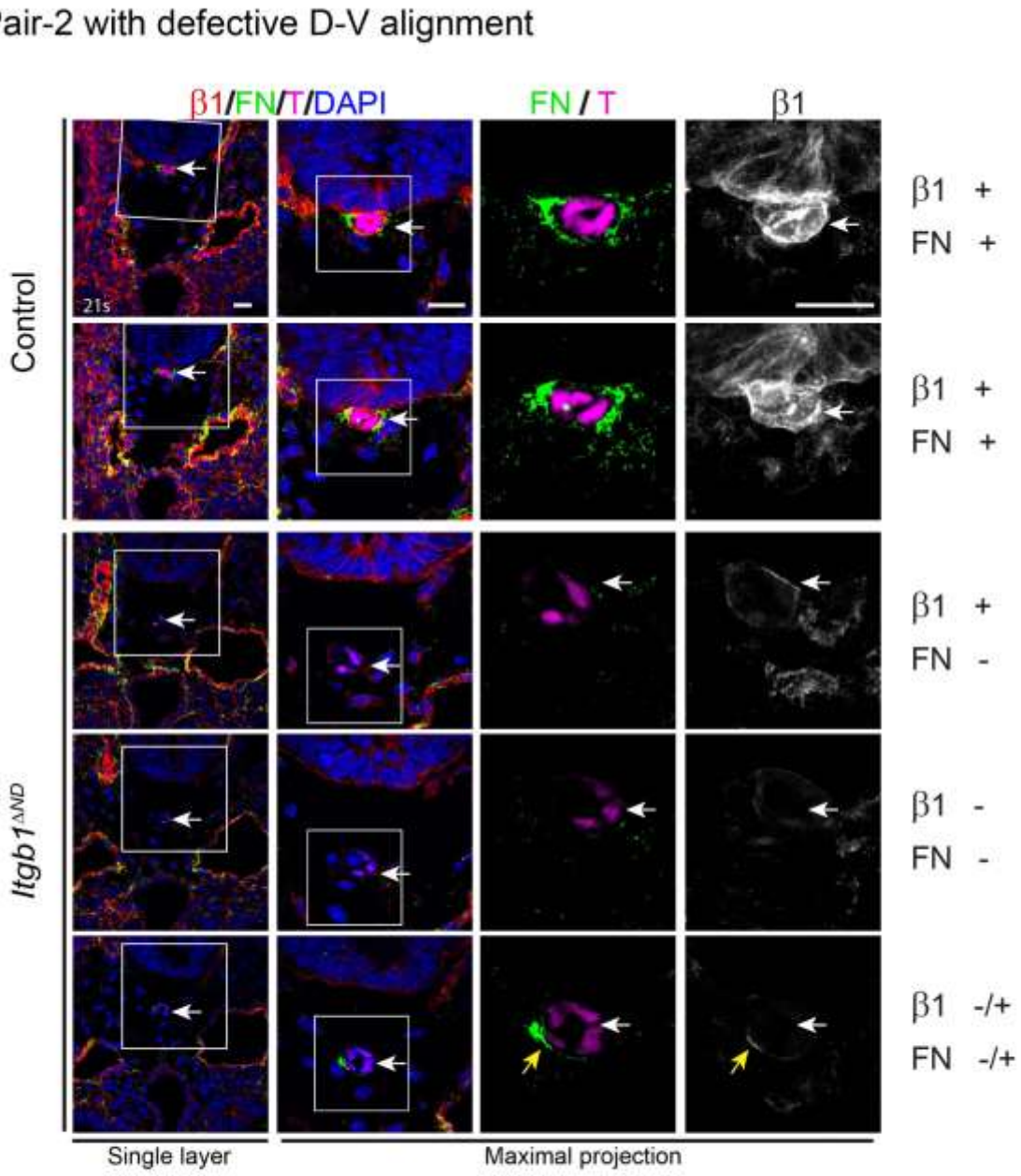


Response Fig. 1 Reduced FN assembly in the mutants. 3 paired analysis were shown (Pair-1 to -3). Immunostaining of the notochord sheath against FN (green), Bra (magenta) and β1 integrin (red) in the cross sections from E9.5 embryos. The correlation between β1 integrin expression and FN matrix assembly were illustrated on the right hand side of each panel. Arrows: notochord. Scale bars: 20 μm.

Response Table 1.
Embryo details for FN sheath formation analysis

Embryo ID.	#1 (21s)	#2 (16s)	#3 (21s)	#4 (22s)	#5 (23s)	#6 (25s)	#7 (23s)
Notochord regions analyzed	8	8	8	8	8	8	8
Notochord alignment	D-V deviation			L-R deviation		normal	
FN loss/reduction	6/8	4/8	6/6	0/5	2/8	0/8	0/8
Notochord missing	0/8	0/8	2/8 (posterior)	3/8 (Anterior + posterior)	0/8	0/8	0/8
Frequency of FN loss/reduction: 4/7= 57%. **#3(21s): the representative images shown in the manuscript (Fig.3J).**							

Response Figure 1-Pair-2



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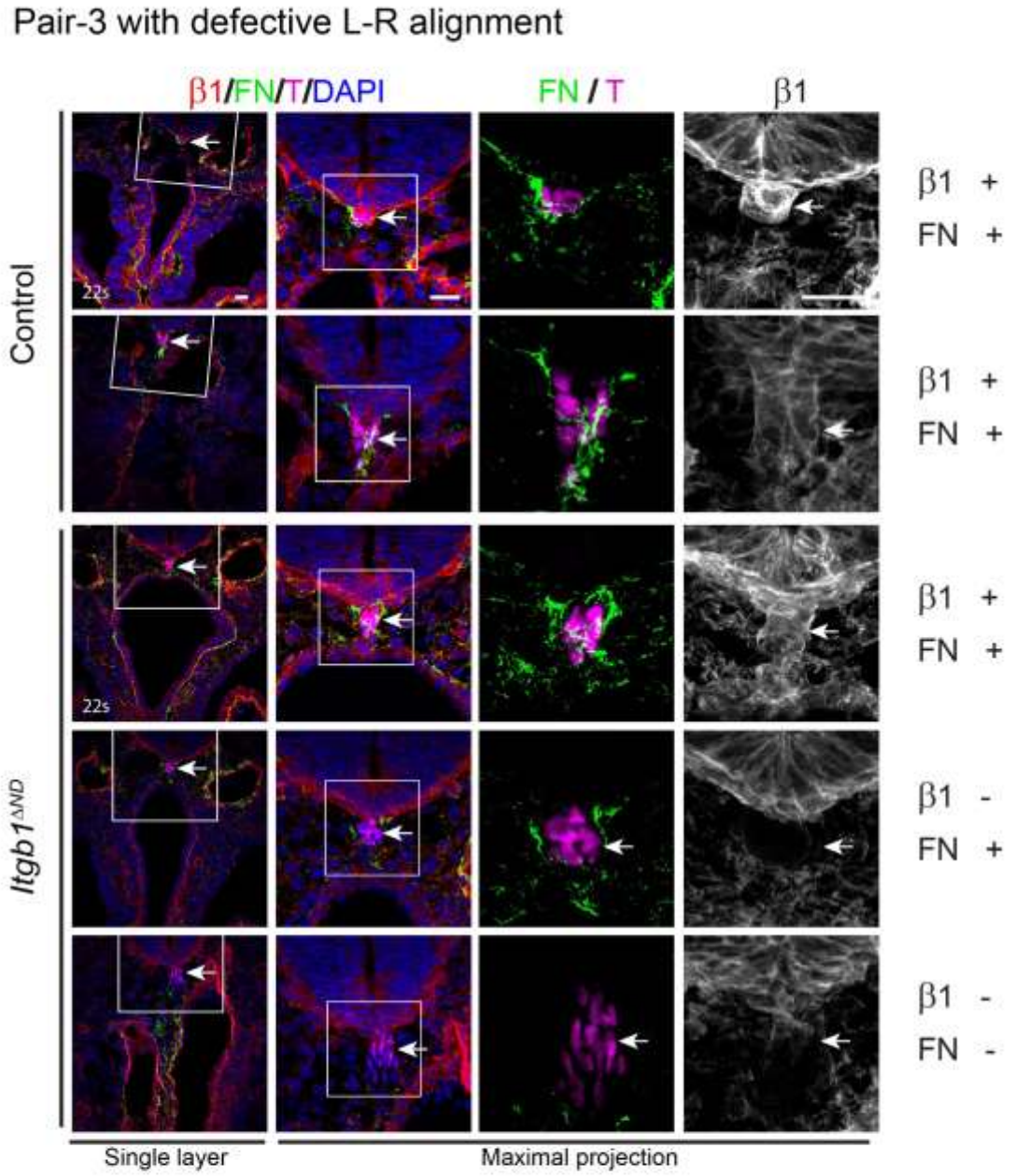
Response Figure 1-Pair-3



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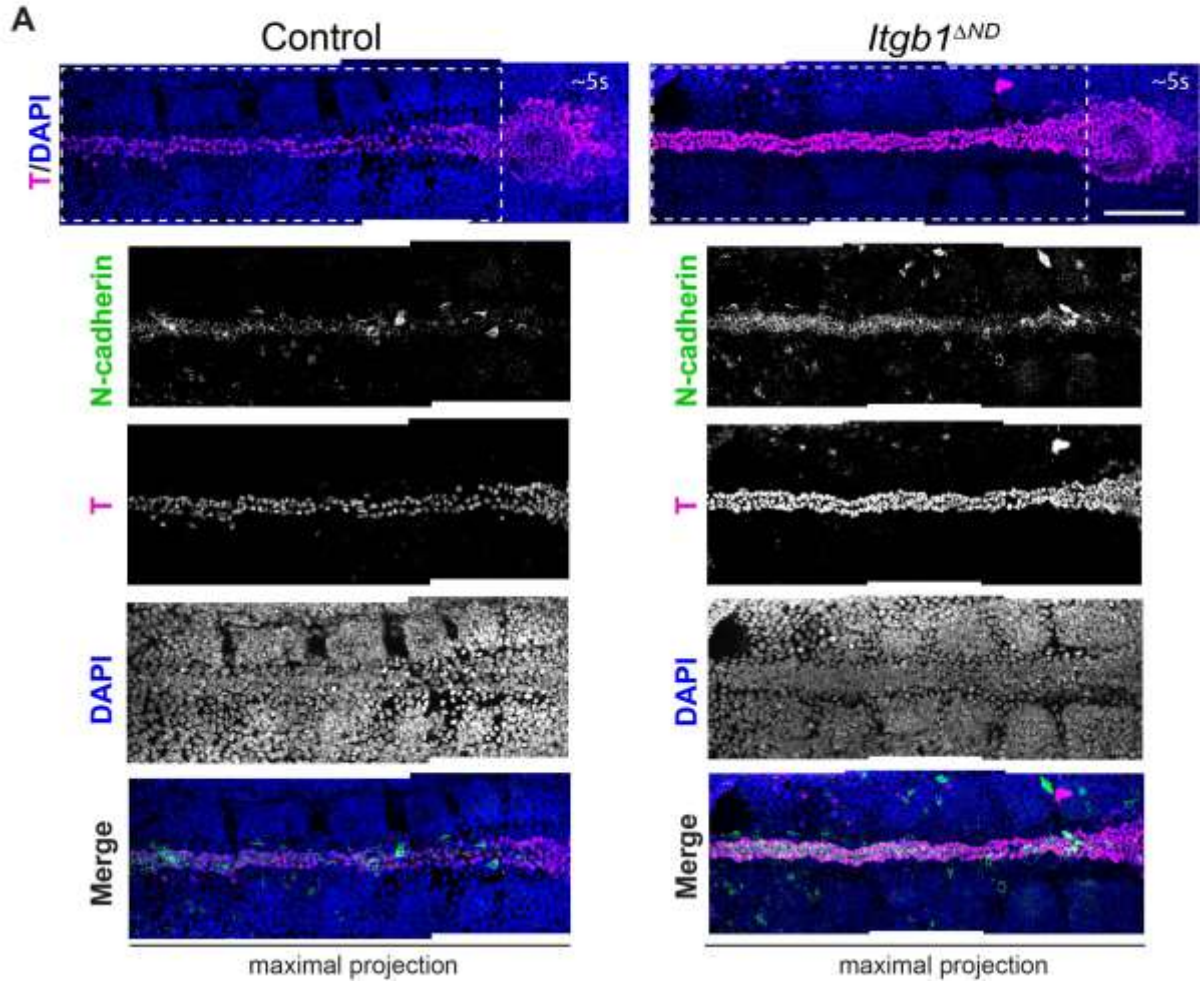
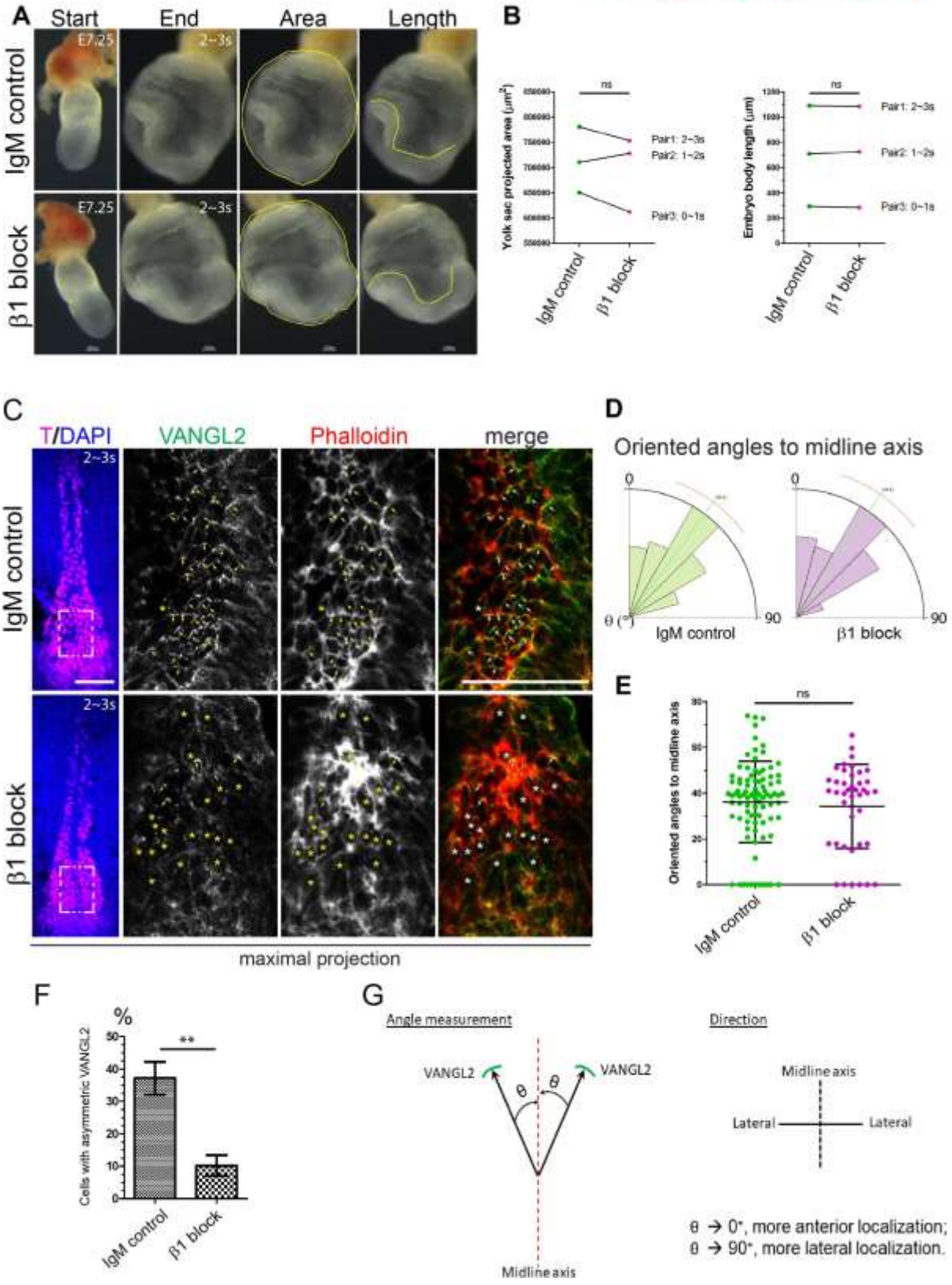
Response Figure 2
(repeat shown in the initial submission)



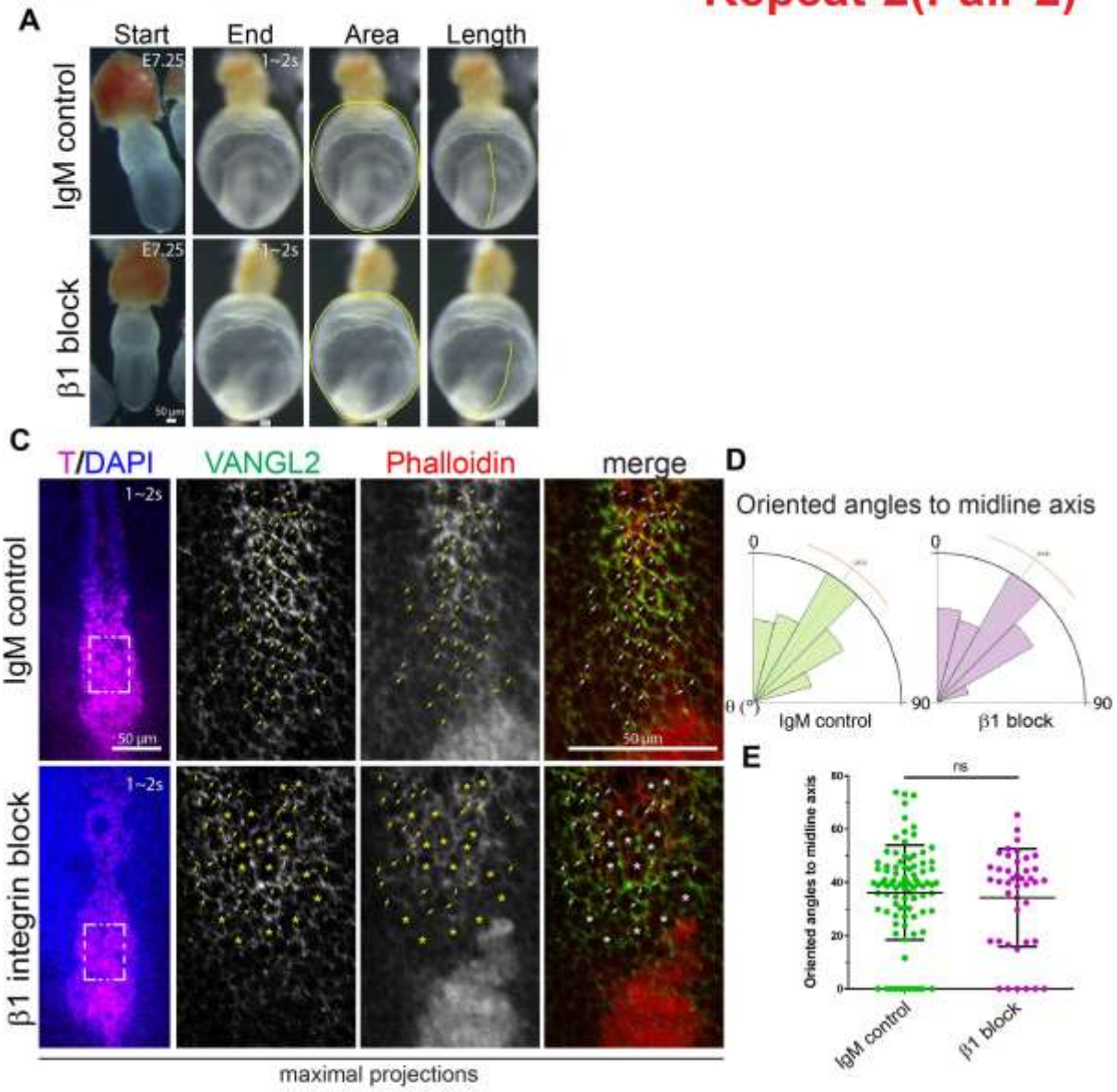
Response Figure 3

Repeat-1(Pair-1)



Response Figure 3

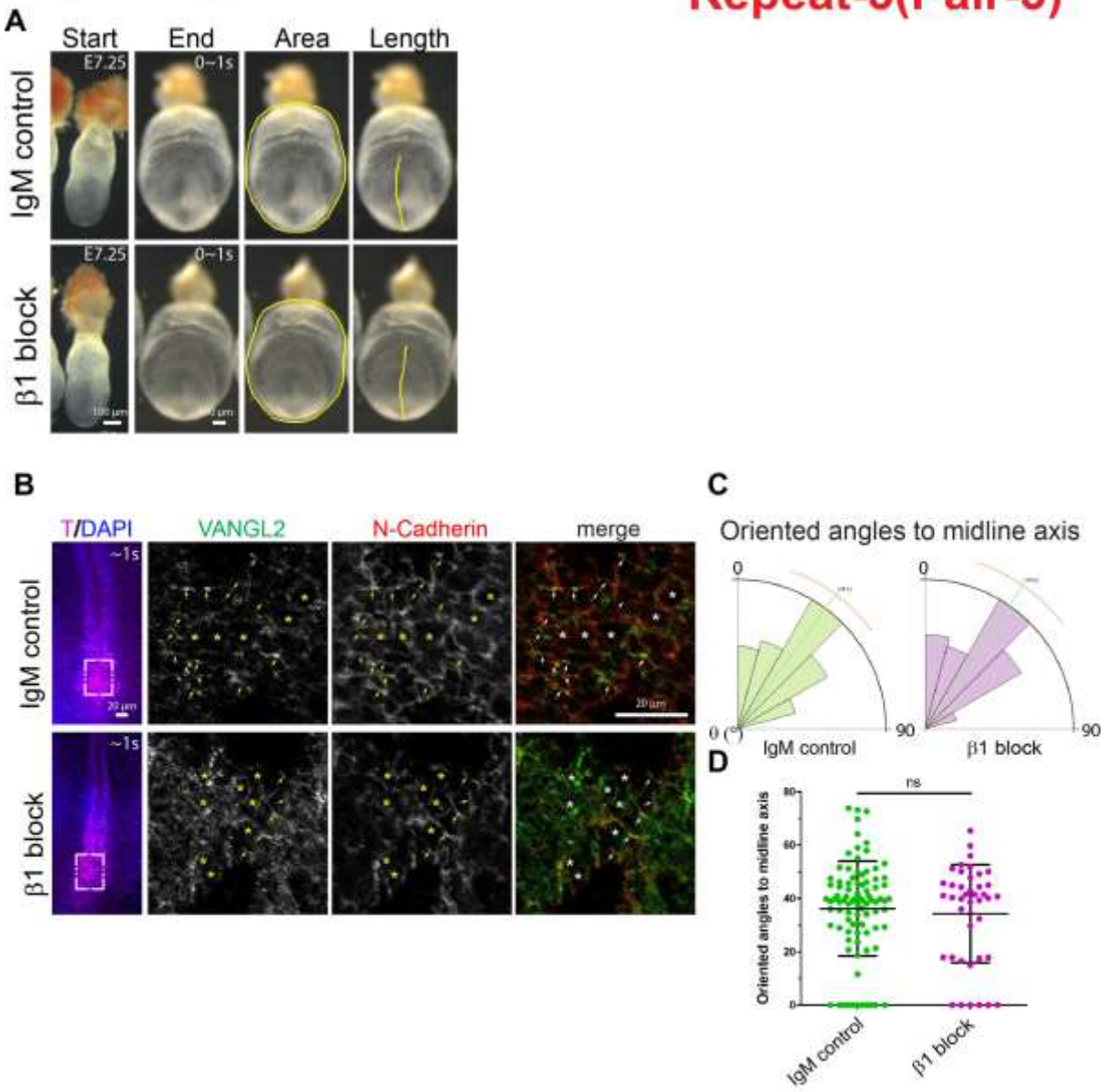
Repeat-2(Pair-2)



Response Fig. 2 Reduced asymmetric VANGL2 localization in the node when β1 integrin function is blocked. 3 paired analysis were shown (Pair-1 to -3).
(A) Embryo morphology before and after ~24 hours rotating culture in the presence of either IgM control or β1 specific functional blocking antibody. Age at the starting point and at the end point were indicated. Embry growth was monitored by measuring the yolk sac projection area and the midline length at the end of culture.
(C) Whole embryo staining against T (magenta), VANGL2 (green), Phalloidin or N-Cadherin (red) and DAPI (blue). VANGL2 asymmetric localization in the node were shown in details. Arrows: asymmetric VANGL2 accumulation. Note this pattern was much reduced in the blocking group. Asterisks: cells without a clear asymmetric VANGL2 accumulation.
(D-E) VANGL2 orientation with respect to midline axis were measured and plotted in ROSW diagram and quantified in D. ns: no significance (two-tailed student t-test).

Response Figure 3

Repeat-3(Pair-3)



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(C-D) VANGL2 orientation with respect to midline axis were measured and plotted in ROSW diagram and quantified in D. ns: no significance (two-tailed student t-test).

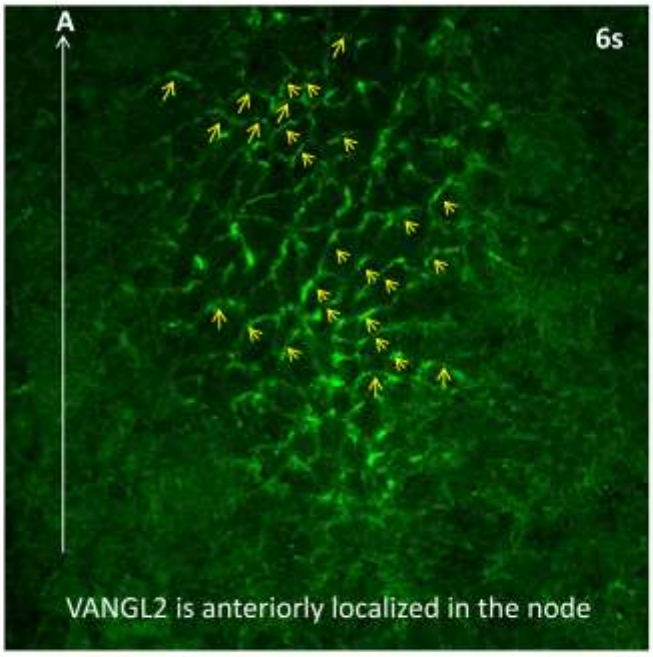
Response Figure 4

PCP markers tested in our hand

Antibody	Source	Cat.num.	Tested tissue	Polarized signal
Vangl1	Yingzi Yang's lab		E8.5 embryo	no
Vangl1	Sigma	HPA025235	E8.5 embryo	no
Vangl2	Matthew W. Kelly"s lab		E8.5 embryo	Clear polarization
Celsr1	Santa Cruz	SC-99197	E8.5 embryo	no
Dvl1	LSBbio	LS-B11264	E13-E14-E18 tibia	no
Dvl2	Biomol	da4270	E13-E14-E18 tibia	no
Fzd6	Sigma	HPA017991	E16-E18 tibia	no
Fzd7	Avivasysbio	ARP41251	E16-E18 tibia	no
Prickle 1	Abcam	ab15577	E13-E14 limb cartilage	no

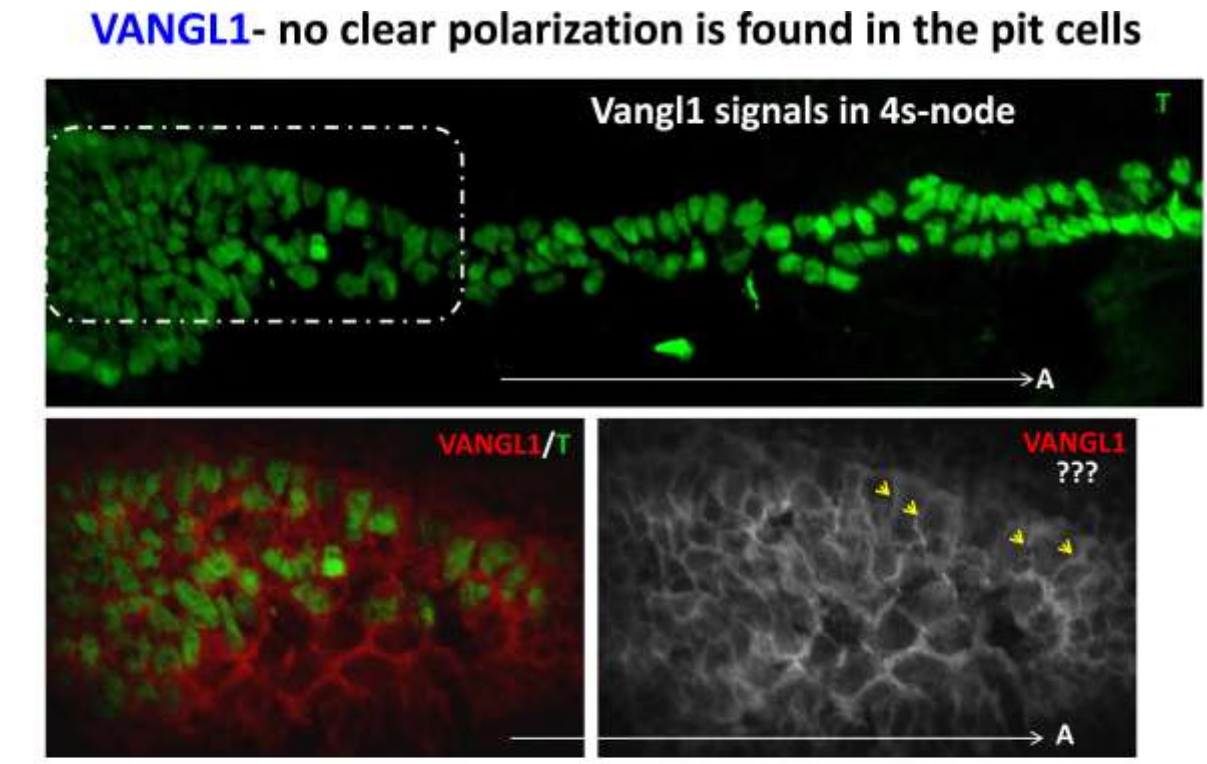
Node in 6s embryo

VANGL2-anteriorly localized in the pits cells



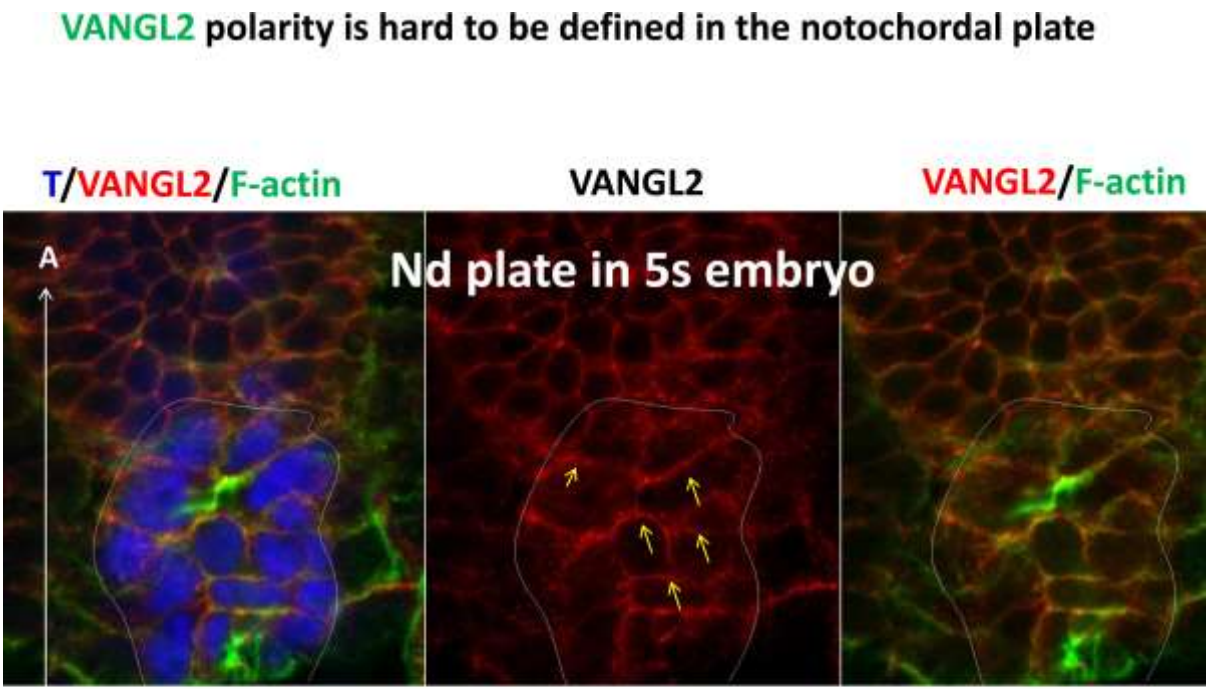
VANGL2 Ab. from Matthew W. Kelly's lab

Response Figure 4



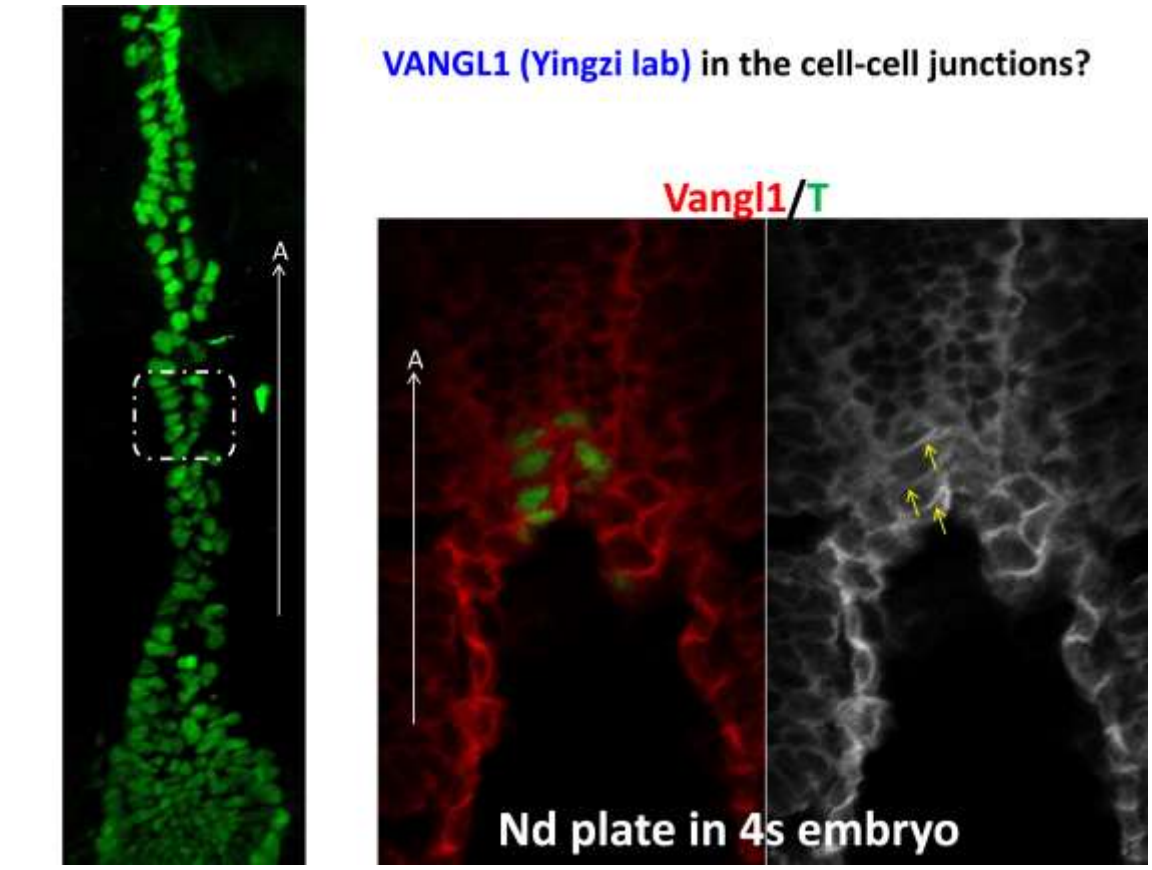
Response Figure 4

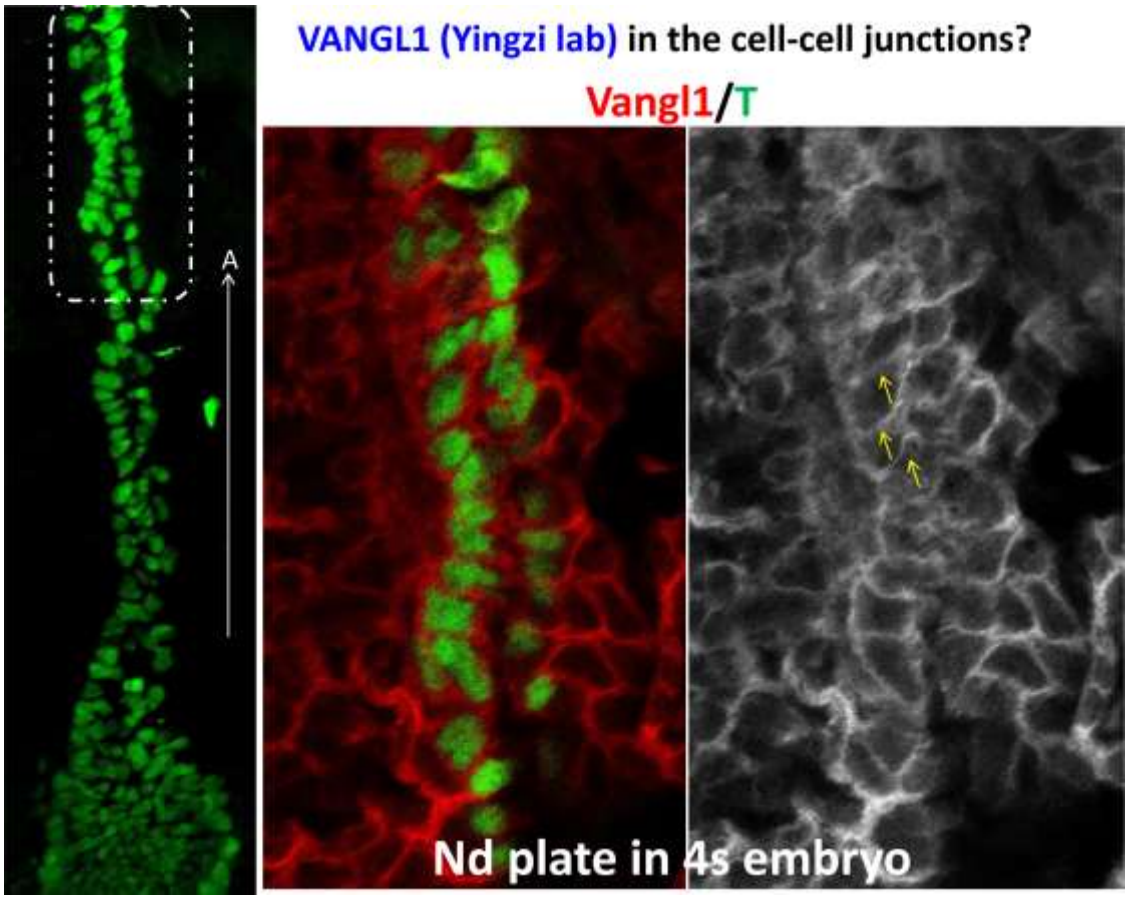
VANGL1 Ab. from Yingzi Yang's lab



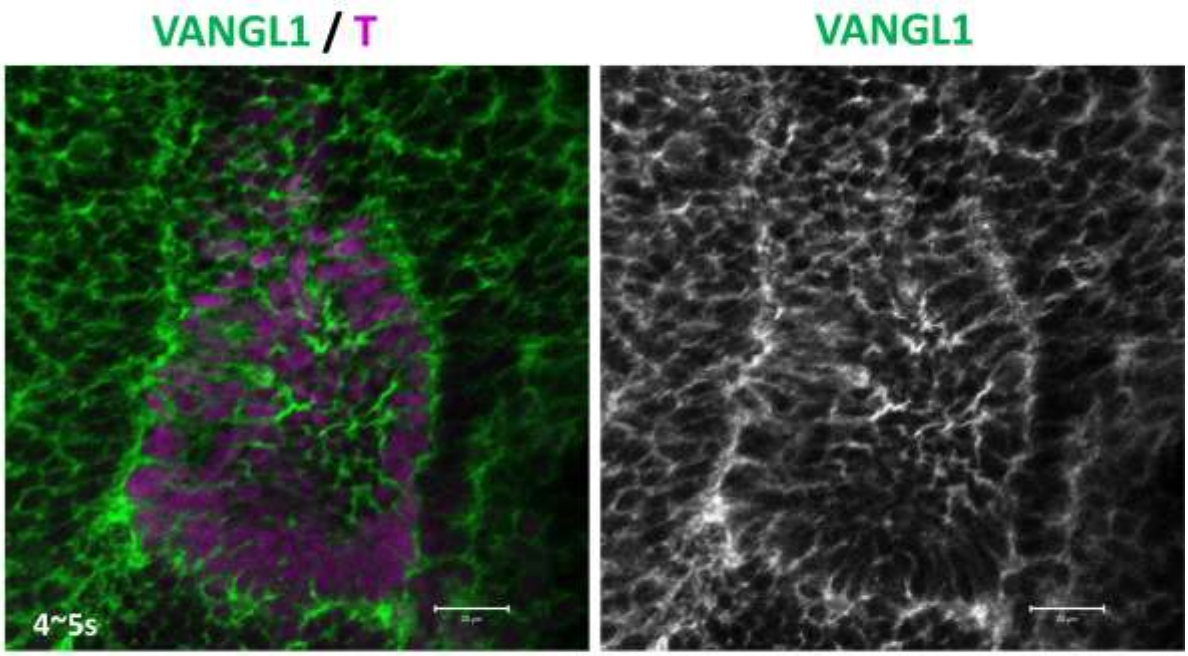
Response Figure 4

VANGL2 Ab. from Matthew W. Kelly's lab





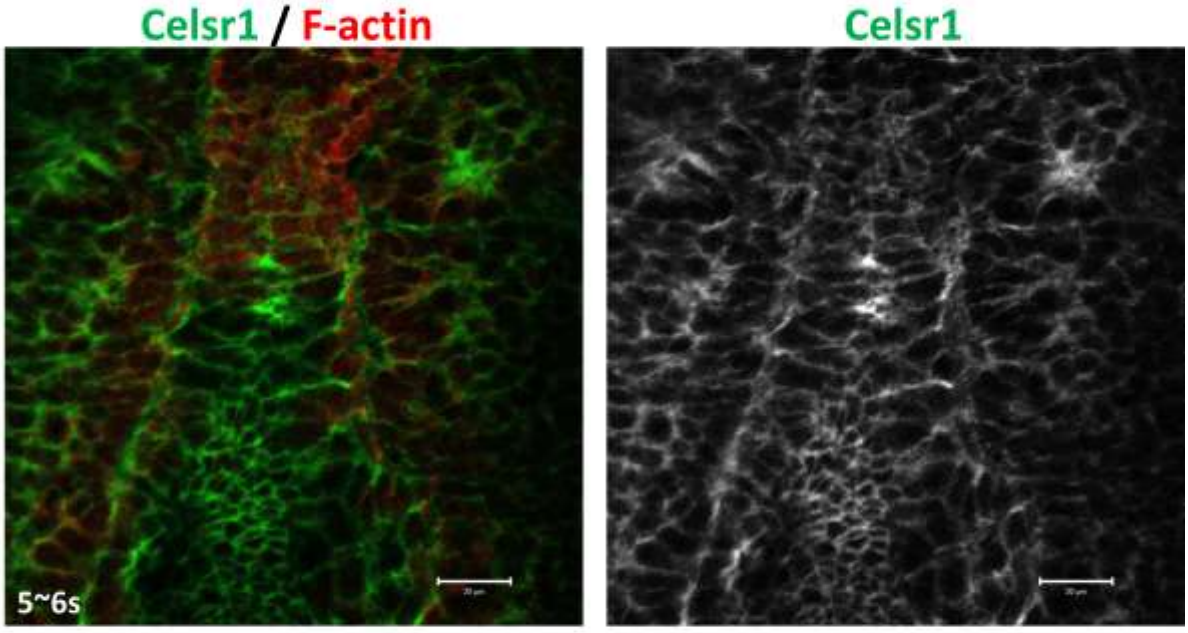
VANGL1 (Sigma): polarity can be defined but less potent than **VANGL2**.



Sigma HPA025235

Response Figure 4

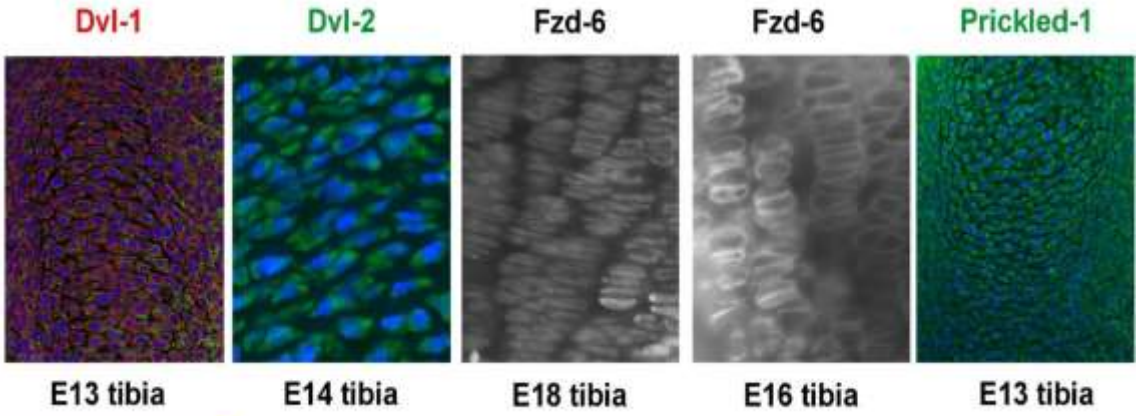
Celsr1 (Santa cruz): polarity can be defined but less potent than **VANGL2**.



Santa cruz SC-99197

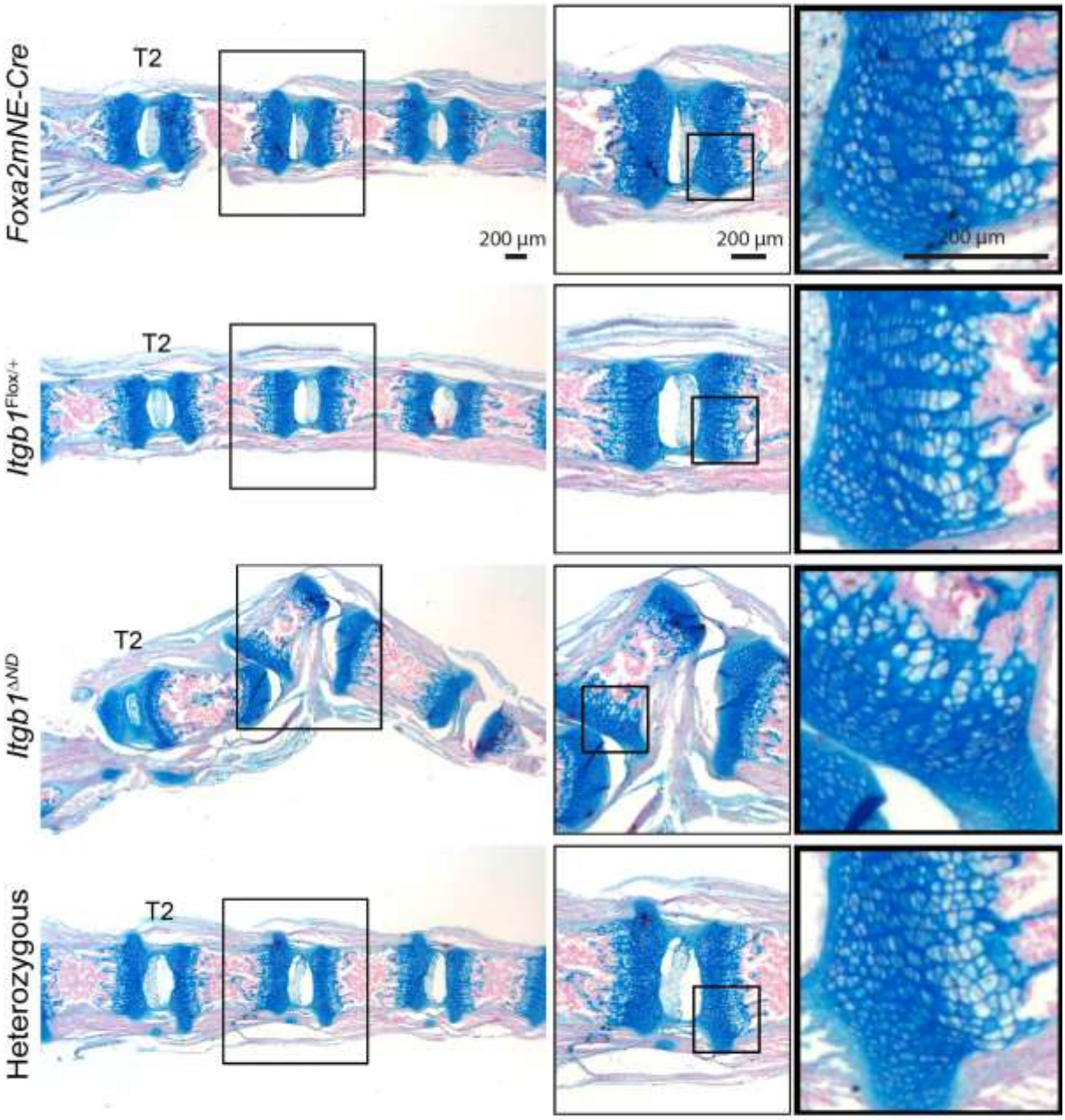
Response Figure 4

Antibody	Source	Cat.num.	Tested tissue	Polarized signal
Dvl1	LSBbio	LS-B11264	E13-E14-E18 tibia	no
Dvl2	Biomol	da4270	E13-E14-E18 tibia	no
Fzd6	Sigma	HPA017991	E16-E18 tibia	no
Fzd7	Avivasysbio	ARP41251	E16-E18 tibia	no
Prickle 1	Abcam	ab15577	E13-E14 limb cartilage	no



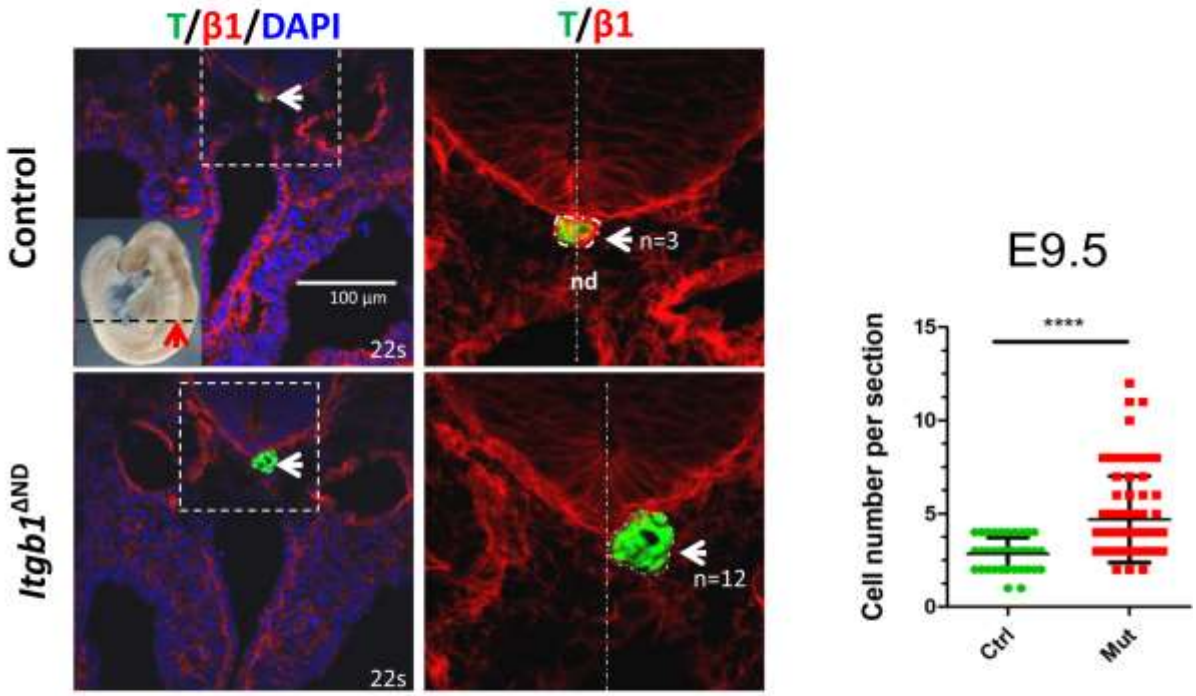
Response Figure 4

Response Figure 5

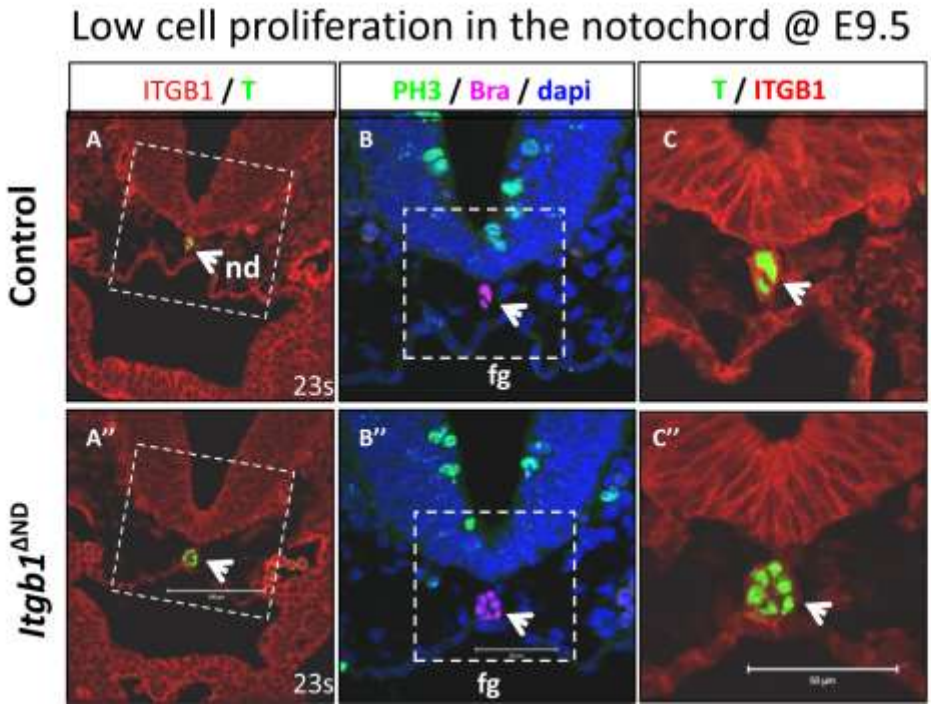


Response Fig. 5 Hypertrophic chondrocytes differentiation were comparable between different genotypes. Histological analysis of the tail vertebra at P10. Vertebral levels shown are between proximal 2 to 4.

Response Figure 6

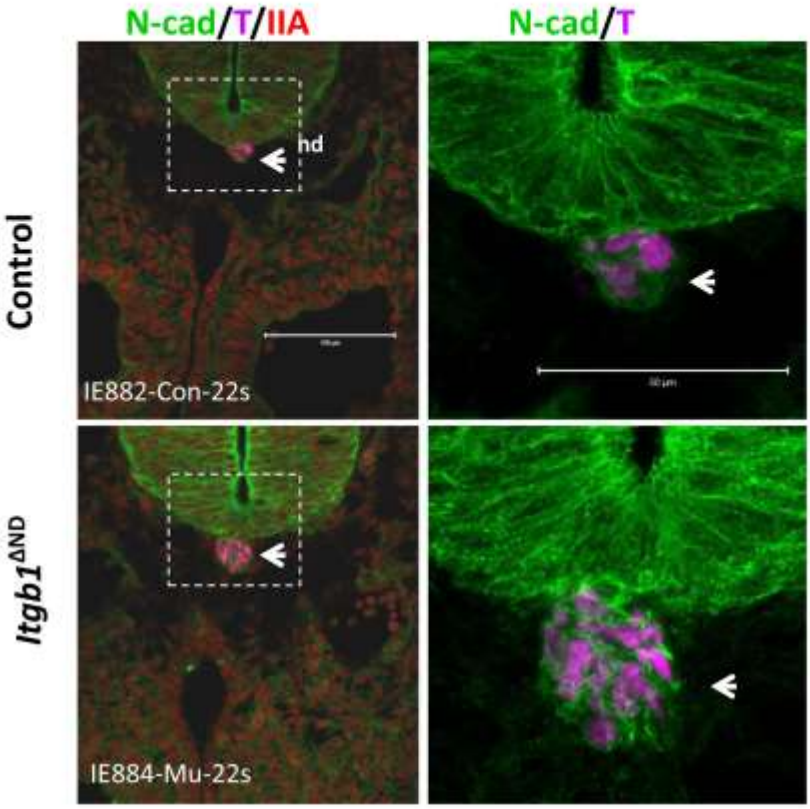


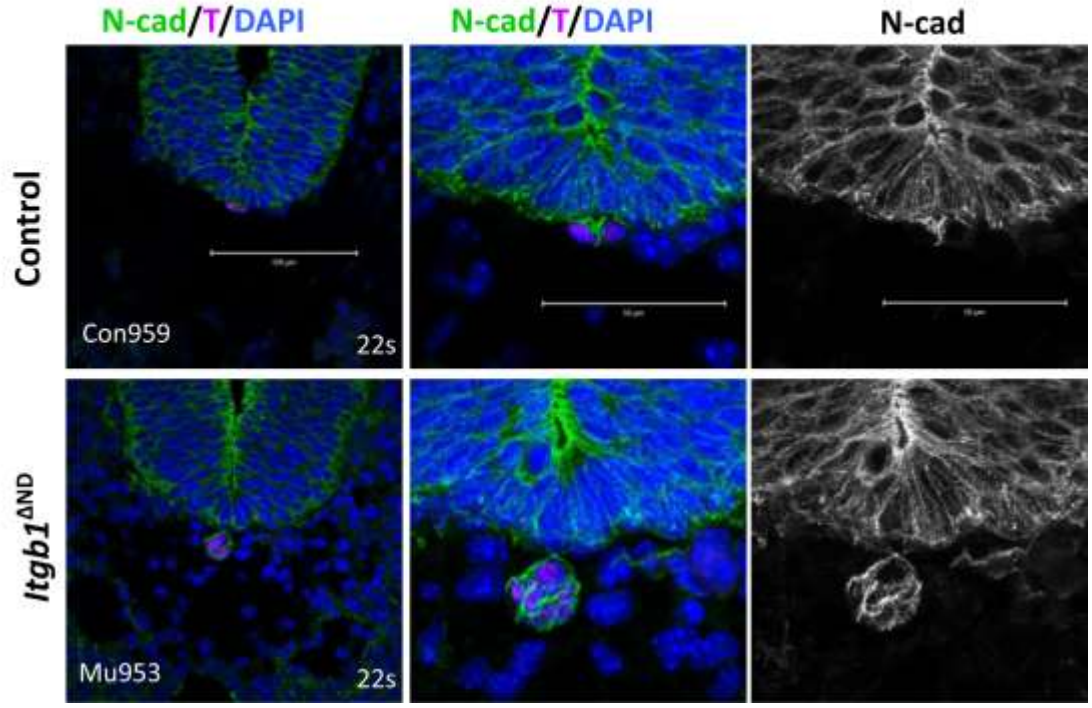
More examples included for cell counting



Response Figure 6

More examples included for cell counting





Second decision letter

MS ID#: DEVELOP/2020/192724

MS TITLE: β1 integrin regulates convergent extension in mouse notogenesis, ensures notochord integrity and the morphogenesis of vertebrae and intervertebral discs

AUTHORS: Shiny Shengzhen Guo, Tiffany YK Au, Sarah Wynn, Attila Aszodi, Danny Chan, Reinhard Faessler, and Kathryn Cheah

I have now received all the referees reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

The overall evaluation is positive and we would like to publish a revised manuscript in Development. As you will see Referee 1 and 2 are satisfied with your revisions; Referee 2 picked up a minor typo that should be corrected. Referee 3, while recognising your revisions have strengthened the manuscript, remains concerned about the blocking antibody experiment. Nonetheless, I think that inclusion of these data are justified. However, it would be helpful to readers if the potential caveat to the interpretation of these data is acknowledged when you discuss the results on p21 of the Discussion.

Please detail your changes in a point-by-point response. If you do not agree with the criticism please explain clearly why this is so.

We are aware that you may currently be unable to access the lab to undertake experimental revisions. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Reviewer 1

Advance summary and potential significance to field

This paper describes the roles of beta1 integrin (Itgb1) in mouse notochord morphogenesis. To avoid peri-implantation lethality, the authors produced notochord-specific deletion mutants of the Itgb1 gene. Approximately half of the mutant mice showed abnormalities in vertebral body, intervertebral discs and nucleus pulposi at birth. In the mutant embryos, some parts of the notochord were fragmented and displaced. The mutant notochord showed multiple abnormalities including loss of fibronectin around the notochord, reduced convergent-extension movement of the node/notochord cells, and increased N-cadherin. Cell proliferation, cell death, and planar cell polarity were not affected. Authors established a notochord cell line, and showed that Itgb1 mutant cells lost attachment to fibronectin and have reduced cell motility.

The authors demonstrated requirement of Itgb1 in notochord development specifically for formation of the fibronectin sheath and morphogenetic cellular movement in mouse embryos. These findings are novel and important for understanding the roles of integrin signaling and integrin-mediated interaction with ECM during morphogenesis. The results are supported by detailed analyses of fixed embryos, high-quality live imaging, and in vitro studies using the newly established notochord cell line.

Comments for the author

In the revised version, the authors appropriately addressed all the issues raised by the reviewers. I think that the paper is suitable for publication.

Reviewer 2

Advance summary and potential significance to field

I found that the authors addressed most of the concerns from the initial review and now present a much improved manuscript.

This paper demonstrates that B1 integrin is necessary to help CE and set up membrane localization of VANGL2, future efforts might explore whether this is a general effect of loss of tissue integrity which generally affects the membrane localization of PCP components or a specific VANGL2 interaction.

The addition of PAX1 in-situ hybridization in whole-mount mouse embryos suggests that the loss of notochord integrity has a signaling role for the paraxial mesoderm/ somites which may further contribute to defects in IVD development, that are in addition to simply losing notochord derived NP tissues. To me this suggests that the notochord is important for patterning the spine in mammals as has been demonstrated in telosts. Thus this work will be foundational for future analysis of this model.

Comments for the author

minor comment:
Line 254: There is no description of the abbreviation of LN (Laminin) in the text.

Reviewer 3

Advance summary and potential significance to field

This manuscript demonstrated that Integrin B1 is required for notogenesis and that its selective deletion causes vertebral malformations. The authors provide evidence that disruption of planar polarised Vangl2 localisation is involved in the development of these phenotypes.

Comments for the author

The authors have made changes to the methods, results and discussion sections which largely address my initial concerns. They provide new data which helps further characterize their model and provides new insights.

I remain unconvinced that the blocking antibody experiment shows changes due to the meaningful localisation of a functional hamster IgM (massive pentamer Fc hidden on the inside of the protein, does not cross the human placenta) in whole-cultured mouse embryos. The references provided do not demonstrate yolk sac absorption and redistribution of functional IgM. I would be more convinced if the authors treated embryos with this antibody in culture then proceeded directly to immunofluorescence using an anti-hamster IgM secondary antibody to demonstrate localisation of the antibody within the notochord. The authors may also wish to provide positive controls specific to the integrin pathway such as pFAK.

The alternative suggested by the authors, removing this data given limitations to its interpretation now discussed, is acceptable but the authors would also need to amend their discussion of the role of Itgb1 in establishment of PCP.

Second revision

Author response to reviewers' comments

Reviewer 1 Advance Summary and Potential Significance to Field...

This paper describes the roles of beta1 integrin (Itgb1) in mouse notochord morphogenesis. To avoid peri-implantation lethality, the authors produced notochord-specific deletion mutants of the Itgb1 gene. Approximately half of the mutant mice showed abnormalities in vertebral body, intervertebral discs and nucleus pulposi at birth. In the mutant embryos, some parts of the notochord were fragmented and displaced. The mutant notochord showed multiple abnormalities including loss of fibronectin around the notochord, reduced convergent-extension movement of the node/notochord cells, and increased N- cadherin. Cell proliferation, cell death, and planar cell polarity were not affected. Authors established a notochord cell line, and showed that Itgb1 mutant cells lost attachment to fibronectin and have reduced cell motility.
The authors demonstrated requirement of Itgb1 in notochord development, specifically for formation of the fibronectin sheath and morphogenetic cellular movement in mouse embryos. These findings are novel and important for understanding the roles of integrin signaling and integrin-mediated interaction with ECM during morphogenesis. The results are supported by detailed analyses of fixed embryos, high-quality live imaging, and in vitro studies using the newly established notochord cell line.

Reviewer 1 Comments for the Author...

In the revised version, the authors appropriately addressed all the issues raised by the reviewers. I think that the paper is suitable for publication.

Response:
We thank the reviewer for the positive comments.

Reviewer 2 Advance Summary and Potential Significance to Field...

I found that the authors addressed most of the concerns from the initial review and now present a much improved manuscript.

This paper demonstrates that B1 integrin is necessary to help CE and set up membrane localization of VANGL2, future efforts might explore whether this is a general effect of loss of tissue integrity which generally affects the membrane localization of PCP components or a specific VANGL2 interaction.

The addition of PAX1 in-situ hybridization in whole-mount mouse embryos suggests that the loss of notochord integrity has a signaling role for the paraxial mesoderm/ somites which may further contribute to defects in IVD development that are in addition to simply losing notochord derived NP tissues. To me this suggests that the notochord is important for patterning the spine in mammals as has been demonstrated in telosts. Thus this work will be foundational for future analysis of this model.

Reviewer 2 Comments for the Author...

minor comment:
Line 254: There is no description of the abbreviation of LN (Laminin) in the text.
Response:
We thank the reviewer for very helpful suggestions from the initial review.
To ensure the writing consistency, we had added the abbreviation of laminin as LN when it first appeared in the text in Page 5 (Line 87).

Reviewer 3 Advance Summary and Potential Significance to Field...

This manuscript demonstrated that Integrin B1 is required for notogenesis and that its selective deletion causes vertebral malformations. The authors provide evidence that disruption of planar polarised Vangl2 localisation is involved in the development of these phenotypes.
Reviewer 3 Comments for the Author...

The authors have made changes to the methods, results and discussion sections which largely address my initial concerns. They provide new data which helps further characterize their model and provides new insights.

I remain unconvinced that the blocking antibody experiment shows changes due to the meaningful localisation of a functional hamster IgM (massive pentamer, Fc hidden on the inside of the protein, does not cross the human placenta) in whole-cultured mouse embryos. The references provided do not demonstrate yolk sac absorption and redistribution of functional IgM. I would be more convinced if the authors treated embryos with this antibody in culture then proceeded directly to immunofluorescence using an anti-hamster IgM secondary antibody to demonstrate localisation of the antibody within the notochord. The authors may also wish to provide positive controls specific to the integrin pathway such as pFAK.

The alternative suggested by the authors, removing this data given limitations to its interpretation now discussed, is acceptable but the authors would also need to amend their discussion of the role of Itgb1 in establishment of PCP.

Response:
We thank the reviewer for the positive comments on the efforts we made during the last 3 months. And for the remaining concern about the IgM antibody culture, as suggested by the handling editor, we have updated this possible caveat into the discussion (Page 20-21, Line 433-441).

Third decision letter

MS ID#: DEVELOP/2020/192724

MS TITLE: β1 integrin regulates convergent extension in mouse notogenesis, ensures notochord integrity and the morphogenesis of vertebrae and intervertebral discs

AUTHORS: Shiny Shengzhen Guo, Tiffany YK Au, Sarah Wynn, Attila Aszodi, Danny Chan, Reinhard Faessler, and Kathryn Cheah
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks.